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Maize (Zea mays L.) kernel development, suitability for dry-grind ethanol production, and susceptibility to aflatoxin accumulation in relation to late-season water stress

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Maize (*Zea mays* L.) kernel development, suitability for dry-grind ethanol production, and susceptibility to aflatoxin accumulation in relation to late-season water stress

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Table of Contents

List of Tables

Chapter 4

List of Figures

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Abstract

Worldwide, water stress during vegetative and reproductive development of cereal species is a primary constraint to crop yield. In maize (*Zea mays* L.), environmental stresses such as high temperature and drought affect yield by reducing the number and mean dry weight of harvested seeds per unit area. Total grain yield is of great economic concern, and increasing yield under optimal and stress conditions has historically received the most attention by public and private breeding programs. But environmental effects on grain quality is of equal importance as maize production becomes more focused on end-user traits specifically for utilization in markets such as dry-grind ethanol, wet-milling, and enhanced animal feeds. While these traits have demonstrated great economic potential, the variability in their corresponding properties as a result of adverse environmental conditions during grain development is not well understood. Further development and utilization of valuable end-user traits will require a greater understanding of kernel development and composition during environmental stress.

In recent years, the production of fuel ethanol from maize grain has increased in response to concerns over dependency on foreign sources of oil, global climate change stemming from the use of petroleum sources of transportation fuel, and the need to stimulate struggling rural economies. While grain to ethanol likely represents a temporary energy conversion technology, there is clearly a need to improve the process by increasing ethanol yield per unit area and unit of feedstock. Two such strategies are to increase total starch yield through improved genetics and agronomic practices, and to increase the conversion efficiency of starch to fermentable sugars. Previous reports of ethanol yield suggest that starch is not always equally digestible across hybrids and environments. Although these reports clearly demonstrate that there is variability in starch hydrolysis and potential for ethanol yield, they do not conclusively identify specific causal traits that can be altered through plant breeding.

The central objective of this thesis was to assess the impact of water stress on maize grain development, composition, and the resulting susceptibility of starch to enzymatic hydrolysis. The results suggest that while the dry weight of B73 maize kernels and contents

of starch, protein, and oil are decreased markedly by water stress, the concentrations of these chemical constituents are not significantly affected. Kernel density and hardness, however, are decreased by water stress resulting in greater ease of grinding and a particle size distribution consisting of a greater proportion of fine particles. It is likely that these fine particles increase the susceptibility of kernel starch to hydrolysis by α-amylase and glucoamylase. Although the efficiency of starch hydrolysis appears to be increased by water stress during kernel development, other negative factors such as reduced yield would be detrimental to the ethanol industry. Therefore, in the interest of ethanol conversion efficiency, plant breeders and crop physiologists should focus on developing maize germplasm with high yield under water stress conditions while preserving some of the beneficial traits exemplified by drought stressed kernels.

In addition to mankind's desire to efficiently utilize starch, other organisms such as fungi might also benefit from easily digestible starch. For example, *Aspergillus flavus*, which produces a carcinogenic mycotoxin known as aflatoxin, invades maize kernels and utilizes starch as a substrate for growth and secondary metabolite production. This fungus typically infects maize kernels under conditions of drought and high temperature. As such, environmental challenges to kernel development might alter kernel composition in a way that favors the fungus and the hydrolytic activities of its enzymes. The results of this thesis demonstrate that in whole kernels of B73, irrigated kernels are actually more susceptible to aflatoxin contamination than drought stressed kernels. Embryo and endosperm wounding supported higher levels of aflatoxin in both treatments compared to surface and un-inoculated kernels indicating that an intact pericarp is an important barrier to fungal infection and subsequent aflatoxin accumulation. Under field conditions, drought has consistently been associated with elevated aflatoxin concentrations in grain so the contradictory result of this study should be investigated in further detail.

Although drought stress is an important challenge to grain production that is receiving increased research attention, the transcriptional and metabolic changes that lead to impaired kernel development are not fully characterized. Further efforts to describe stress-specific responses to the environment will require methods for comparing genotypes and treatments under controlled stress environments. The use of kernel moisture content as a

means of comparing kernel dry weight accumulation across genotypes and treatments has previously been demonstrated by other studies. This approach clearly indicates that although the rate and duration of grain filling can vary for different genetic and source-sink treatments, key developmental transitions usually occur at similar moisture content values. As such, this thesis sought to determine whether endosperm transcriptional programs in irrigated and water-stressed kernels follow changes in kernel water content. A preliminary analysis of three genes involved in starch and protein metabolism indicate that gene expression generally declines following maximum kernel water content. Gene expression does not reach low levels, however, until after physiological maturity suggesting that gene expression *per se* might not be limiting to the duration of grain filling. Of particular interest is the transcriptional response of the gene encoding for the 19-kD α-zein protein. A large peak in relative mRNA abundance in irrigated kernels was measured at approximately 30 days after pollination while relative mRNA abundance in water stressed kernels is declining. This result may support the lower density and vitreousness observed in water stressed kernels.

Chapter 1. General Introduction

Thesis Organization

This thesis is divided into five chapters. These include a general literature review, three manuscripts, and general conclusions. The literature review provides general information for the three main areas of this thesis including: i) the maize kernel developmental pattern and perturbations to this pattern attributable to abiotic stresses, ii) maize kernel storage product biosynthesis and properties, iii) maize starch digestibility and relationship to variability in dry grind ethanol production, and iv) aflatoxin accumulation in maize. Following the literature review three manuscripts are included. The first describes the development of maize kernels grown under irrigated and water-stressed greenhouse conditions and the resulting impact on kernel composition and starch digestibility properties. The second manuscript describes differences in aflatoxin accumulation between irrigated and water-stressed kernels with discussion on the potential influence of kernel composition and susceptibility of kernel starch to hydrolysis on aflatoxin accumulation. The final manuscript examines the expression of three genes related to maize starch and protein biosynthesis in relation to changes in kernel water content. Finally, the general conclusions tie together the overall theme of kernel development under water stress conditions and how perturbed development affects kernel size, grain quality, and food safety.

Literature Review

i) Maize kernel development

Botanically, the maize kernel is classified as a caryopsis and is characterized by being a dry, indehiscent, single-seeded fruit at maturity (Watson, 2003). The kernel, which represents a single reproductive unit, is a result of the union of the male gametophyte produced in the pollen grain and the female gametophyte produced in the embryo sac. At flowering, the silks of the female inflorescence (the ear), each leading to an individual

embryo sac, intercept pollen grains produced by the male inflorescence (the tassel). Each pollen grain contains three nuclei; two generative nuclei and one vegetative nucleus. One of the generative nuclei fuses with the egg cell contained within the embryo sac leading to the formation of a 2n zygote. The other generative nucleus fuses with two polar nuclei within the embryo sac to initiate development of triploid (3n) endosperm tissue.

This endosperm tissue is the site of starch synthesis and comprises much of the dry weight (~83%) of the kernel at maturity (Zuber and Darrah, 1987). Of this endosperm, 86-89% by weight is starch and the remaining fraction consists of proteins (~8.0%), lipids (~0.8%), and other miscellaneous compounds (Watson, 2003). Following pollination, kernel development is often described by three phases (Saini and Westgate, 2000). Phase one, the phase of histodifferentiation, is characterized by cellular division and expansion coinciding with a rapid increase in kernel fresh weight (Borrás and Westgate, 2006). This increase in fresh weight is attributed to a rapid change in water content. The number of cells within the endosperm is established at this time, determining the potential or sink capacity for starch accumulation within the amyloplasts contained within the endosperm cells (Reddy and Daynard, 1983; Jones et al., 1996). The majority of the sink capacity is established by approximately 15 days post-pollination. The second phase of kernel development, characterized by rapid dry matter accumulation, begins and continues until approximately 40-50 days post-pollination (Doehlert et al., 1994). At the beginning of the second phase of kernel development, sugar content of the endosperm is relatively high and declines as starch synthesis increases (Ou-Lee and Setter, 1985). As dry matter accumulation progresses, the water content of the kernel decreases until approximately 36% moisture content (fresh weight), at which point the maximum kernel dry weight is reached (Borrás and Westgate, 2006). Beyond this point, an abscission layer commonly referred to as “black layer” forms and further drying occurs as the kernel acquires desiccation tolerance. Perturbations to this general developmental program clearly limit kernel size. Kernel composition might also be altered as a consequence of the transcriptional or metabolic processes that lead to reduced kernel size. As such, it is important to understand the impact of an environmental stress such as water deficit on kernel development and composition if specific quality characters are desired by an end-user of maize grain.

In maize, vegetative growth, pollen viability and silk emergence, early kernel development, and grain filling are negatively affected by water stress to varying degrees (Saini and Westgate, 2000; Boyer and Westgate, 2004). Low water potentials during early kernel development result in increased kernel abortion and decreased kernel size. The latter outcome results from inhibited cellular division within the endosperm leading to diminished sink capacity (Saini and Westgate, 2000). Water stress during grain filling shortens the duration of primary reserve deposition (e.g. starch), which again limits kernel size as measured by final dry weight (Westgate, 1994; Boyer and Westgate, 2004).

The degree to which starch biosynthesis is impaired and starch properties are influenced by low water potentials during rapid dry matter accumulation and prior to the initiation of maturation and drying is unclear. Studies such as those of Zinselmeier et al. (2002) and Yu and Setter (2003) examined phase one of kernel development (histodifferentiation) so their results may not be directly transferable to later stages of development. Zinselmeier et al. (2002) used cDNA microarray technology to measure the expression of genes involved in the starch biosynthetic pathway when maize plants were subjected to low water potential. Similarly, Yu and Setter (2003) studied the gene expression profile of endosperm tissue after maize plants had been subjected to water stress. Although a large number of genes were differentially expressed between well-watered and stressed conditions in both studies, no altered expression was observed for genes involved in starch synthesis. These studies suggest that starch biosynthesis is not directly impacted by water stress and that the early termination of starch accumulation in response to water stress is likely a result of other transcriptional and metabolic responses.

Anatomically, endosperm is vascularly discontinuous from the rest of the plant so the endosperm may not experience the same water potential as the plant (Zinselmeier et al., 2002; Yu and Setter, 2003). Indeed, several studies have demonstrated that the kernel water potential during drought conditions remains stable while the water potential in vegetative structures decreases markedly (Westgate and Thomson Grant, 1989; Westgate, 1994). In contrast to Zinselmeier et al. (2002) and Yu and Setter (2003), other studies have shown that water deficit does influence maize endosperm development through altered enzymatic activity and gene expression. Ober et al. (1991) examined the transcriptional responses and

activities of sucrose synthase and granule bound starch synthase (GBSS) during water stress between 1 and 15 days post-pollination. Sucrose synthase activity was unaffected while mRNA abundance decreased indicating possible post-transcriptional regulation of sucrose synthase activity. Both GBSS enzyme activity and mRNA abundance were significantly decreased, particularly in kernels within the apical region of the ear. The results from analysis of GBSS transcription and activity indicate regulation at the transcriptional level. Ober and colleagues (1991) also assessed the contribution of each enzyme to starch accumulation and found that the activity of sucrose synthase exceeded the starch deposition rate. Apparently enzyme activity was not limiting. The activity of GBSS was less than the rate of starch accumulation, so other starch synthases likely were responsible for the flux of carbon into starch. If so, amylopectin synthesis may have increased as a response to water deficit at this early stage of kernel development (1-15 days post-pollination) since other starch synthases are responsible for amylopectin synthesis, while GBSS is primarily responsible for synthesis of amylose.

In summary, a severe water deficit during the effective grain fill period leads to an early termination of kernel growth limiting kernel size. The specific physiological mechanisms that initiate this response are not well characterized, and further research is needed to understand the impact of perturbed kernel development on grain composition.

ii) Starch synthesis and structure

From the perspectives of maize yield and grain composition, starch is the most important component comprising approximately 70% of the kernel dry weight (John, 1992). Maize starch is an important ingredient in human and animal diets and is also used as a feedstock for a host of industrial products (James et al., 2003). These products include fuels, adhesives, thickening agents, and coatings for papers and textiles (Roper, 2002). Considering the diverse applications of maize starch, there is considerable interest in gaining new insight into the biology of maize seed development and starch biosynthesis so that maize varieties can be developed for specific end-uses such as ethanol conversion efficiency.

Starch synthesis and structure are determined by the concerted action of a number of enzymes. Sucrose generated from photosynthesis in the source leaf is cleaved by invertase

or sucrose synthase to glucose and fructose. A number of enzymes then catalyze isomerization and phosphorylation to yield glucose-1-phosphate. Glucose-1-phosphate is the substrate for ADP-glucose pyrophosphorylase (AGP); the first committed enzyme in the starch biosynthetic pathway. AGP utilizes glucose-1-phosphate to yield pyrophosphate and ADP-glucose (ADPG). This reaction occurs in the cytosol of endosperm cells and ADPG is transported across the amyloplast membrane by a protein encoded by the *Brittle-1* (*Bt1*) locus (Sullivan and Kaneko, 1995; Shannon et al., 1998).

Once inside the amyloplast, ADPG serves as the glucosyl donor for the growing starch polymer. Starch consists of two types of macromolecular glucose polymers. Amylose is an essentially linear molecule of α-1,4 linked glucose subunits. Amylopectin is also comprised of α-1,4 linked glucose subunits but with periodic α-1,6 linked branches. Amylose and amylopectin contribute at the most basic level of starch structure. The proportion of these two starch components in mature kernels varies with the growth conditions of the plant, developmental age, and hybrid (Fergason and Zuber, 1962; Shannon and Garwood, 1984). Typically, however, normal maize genotypes exhibit starch consisting of approximately 25% amylose and 75% amylopectin. Different starch synthases (SS) catalyze the polymerization of the glucosyl subunits derived from ADPG. Granule bound starch synthase I (GBSSI) encoded by the *Waxy* locus is the only enzyme required for amylose synthesis. Several soluble starch synthases (SSI, SSIIa, and SSIII) have been implicated in amylopectin synthesis. The various soluble forms of SS involved in amylopectin synthesis are thought to be specific to conferring various branch chain lengths (James et al., 2003).

As the starch biosynthetic enzymes function within the amyloplasts of endosperm cells, starch granules form from the apposition of additional glucosyl residues from ADP-glucose. Starch granules first appear at approximately 5 DAP (days after pollination) and increase in number until 12 DAP (Li et al., 2007). Throughout the development of the endosperm, the size of these starch granules increases (Jennings et al., 2002; Li et al., 2007). Temporally, amylopectin synthesis is initiated first and amylose content increases throughout reserve deposition indicating that its synthesis is delayed (Martin and Smith, 1995). Li et al. (2007) noted that endosperm amylose content was low until 12 DAP and increased to a

maximum at 45 DAP. This would suggest that smaller, less mature starch granules contain less amylose. Indeed, amylose content in a maize starch granule increases with distance from the center of the mature granule (Pan and Jane, 2000). Amylopectin characteristics also change with time. Li et al. (2007) found that the average chain length of amylopectin branches was greatest at 14 DAP and decreased slightly as the kernel matured.

The proportion of amylose and amylopectin and the arrangement of these molecules within the starch granule influence the physicochemical properties of starch and its utilization. For example, amylose forms helical complexes with lipids and other starch molecules (Seneviratne and Biliaderis, 1991). These complexes limit the access of hydrolytic enzymes. As such, amylose has been negatively correlated with native starch digestibility (Fuwa et al., 1977; Jane, 2006). Other starch properties that are influenced by structure include thermal properties and viscosity characteristics (Ji et al., 2003).

Water stress related perturbations at any point in the starch synthesis pathway might potentially influence resulting starch structure and properties. Although relatively simple in its design, this pathway can clearly generate a diverse array of structural combinations and resultant properties. As natural differences in expression patterns of the starch biosynthetic enzymes suggest a dynamically changing starch structure during kernel development (Li et al., 2007), stress induced changes in expression pattern and activity might also confer altered structure. Understanding the environmental effects on the regulation of starch biosynthesis is essential for designing agronomic practices and plant breeding programs targeted at developing maize starches with specific functional properties.

iii) Storage protein synthesis and properties

During kernel development, proteins accumulate comprising approximately 17.3-19.0% of embryo dry weight and 6.9-10.4% of endosperm dry weight in hybrid maize (Earle et al., 1946). Clearly, many proteins participate in metabolic processes while other proteins are thought only to provide nitrogen for the germinating seedling. The endosperm is the primary site of storage protein synthesis, accumulating prolamins (zeins) and glutelins. Zeins constitute approximately 44% of endosperm protein while glutelins represent 28% of endosperm protein (Hoseney, 1994).

Within the prolamin or zein class of storage proteins, there are many subclasses encoded for by single and multi-gene families. The regulation of zein synthesis has primarily been attributed to transcriptional processes (Kodrzycki et al., 1989). Specifically, zeins are divided into four distinct (α-, β-, γ-, and δ-) subclasses. These subclasses exhibit temporally specific gene expression patterns and are thought to fulfill different roles in the microstructure of the maize endosperm. γ-Zeins along with β-zeins are synthesized first and provide a framework for developing proteins bodies (Lending and Larkins, 1989). α-Zeins represent the largest proportion of the alcohol-soluble storage proteins comprising approximately 70% (Thompson and Larkins, 1989) and fill in the initial protein matrix along with δ-zeins. Further γ- and β-zein synthesis or redistribution occurs later in development and results in a layer of these proteins external to the protein body (Holding and Larkins, 2006).

Kernel hardness is an important characteristic of maize grain related to milling qualities, susceptibility to fungal deterioration, and breakage susceptibility (Chandrashekar and Mazhar, 1999). Maize kernels consist of soft, floury endosperm and hard, vitreous endosperm. The hard endosperm contains a greater proportion of zein protein bodies which form a rigid matrix in association with starch granules. The proportions of each of these types of endosperm are influenced by genotype and environmental conditions (Watson, 2003). For example, flint maize contains a greater proportion of hard endosperm in comparison to dent maize. Additionally, agronomic practices such as nitrogen fertility affect the amount of hard endosperm present (Hamilton et al., 1951). The biochemical basis for kernel hardness is not completely understood but several studies indicate that the 27 kDa γ-

zein is positively correlated with maize grain vitreousness (Mestres and Matencio, 1996; Robutti et al., 1997).

Clearly, assembly of protein bodies within maize endosperm is a complex process. As such, environmental perturbations to kernel development that affect the rate or duration of grain filling might alter the relative composition of individual zein subclasses. Therefore, differences in zein accumulation could influence the hardness of maize kernels.

iv) Variation in grain composition attributable to environment

Clearly, there are genotypic differences for accumulation of starch, protein, and oil in maize kernels. A diverse collection of maize inbred lines grown in Ames, IA during the summer of 2006 illustrates this point (Table 1). Starch concentration ranged from 67.2% to 70.6%, protein concentration from 11.3% to 15.6%, and oil from 3.4% to 5.9%. Also, maize breeders have selected for extreme grain composition traits as exemplified by the long-term selection experiment for high-protein and high-oil at the University of Illinois (Dudley and Lambert, 2004). The deviation from these genetically determined values as a result of environmental challenges to kernel development are not as well documented. Earle (1977) reported the protein content (% dry basis) of maize grain from various sources from 1907 through 1972. Although the quality of the data limited meaningful conclusions, relationships between temperature and rainfall during maize reproductive development were suggested. An inverse relationship between mean July rainfall and protein content was noted as well as a positive relationship between mean July temperature and protein content. The association between reduced rainfall and increased protein content is supported by the results of Harrigan et al. (2007) in which oil and starch concentrations were decreased by a water stress while protein levels increased slightly. Taken together, these studies suggest that an environmental stress that limits kernel development might increase grain protein concentration.

Several studies have reported variation in starch properties associated with grain production across different environments, but few if any have examined the metabolic mechanisms underlying observed differences. A recent study on the influence of various physical and chemical composition traits on digestibility of maize grain as a feed component

for feed lot cattle revealed a hybrid x field interaction for amylose content (Jaeger et al., 2006). The differences in amylose content between fields for the seven hybrids studied ranged from 0.6% to 5.2%. Fergason and Zuber (1962) also reported a genotype x environment interaction for amylose content for commercial dent maize hybrids and maize endosperm mutants. The interaction was most notable for the hybrids and mutants with the greatest genetic potential for amylose synthesis. Observed differences in amylose content were attributed to climate; the lowest amylose content was generally observed in environments with high mean temperature during grain filling. Because amylose content increases later in kernel development (28-35 days after pollination per Ng et al., 1997), the differences observed by Fergason and Zuber (1962) may have resulted from early termination of amylose accumulation induced by thermal stress, which led to reduced potential for amylose accumulation. Support for this hypothesis is evident in Lu et al. (1996) who examined starch structure and properties from kernels of two inbred lines grown at 25^{o}C and 35^{o}C. In addition to reduced grain yield, kernel weight, and kernel density, growth at the higher developmental temperature decreased amylose content 2.2% to 2.4%, relative to controls at 25^{o}C. Amylopectin structure also contained longer branch chains at the higher developmental temperature, and consequently, starch gelatinization temperature increased, as has been observed in other studies (Lenihan et al., 2005). These studies provide clues as to how starch accumulation and properties might be influenced by water deficit during grain filling since high temperatures often accompany drought conditions.

There is ample evidence that environment influences grain composition, starch structure, and the physicochemical properties of maize kernels. The mechanisms through which accumulation of starch, protein, and oil is perturbed, however, are not well understood. Understanding these mechanisms and the limitations they pose to endosperm development is essential to improving the utility and economic value of maize grain.

v) Starch digestibility and ethanol production

With the recent surge in interest in converting corn grain to ethanol (via dry-grind ethanol processing), there have been considerable efforts to characterize the suitability of individual maize hybrids for ethanol yield. Studies at the University of Illinois clearly demonstrate that not all hybrids are equally suitable for use in ethanol production and that ethanol yields for a given hybrid may vary across production environments (Singh and Graeber, 2005; Sharma et al., 2006a). Since controlled fermentation conditions were employed in these studies, ethanol yield actually represents the digestibility of the starch by α-amylase and glucoamylase. These enzymes are used by ethanol plants to hydrolyze starch to sugars supplied to yeast to produce ethanol. In an effort to minimize process variability and maximize efficiency, seed companies such as Pioneer Hi-Bred, Monsanto, and Syngenta have identified and are marketing corn hybrids more suitable for the dry-grind ethanol process. Although it is not publicly known how these hybrids are different than hybrids with average or poor suitability for ethanol production, they are marketed as being "high in total fermentables" and are reported to increase ethanol output by approximately 4% when used in a typical dry-grind ethanol facility (Bothast, 2005).

On average, one bushel of maize grain produces approximately 2.8 gallons of ethanol from the dry-grind process (Bothast and Schlicher, 2005). The theoretical ethanol yield per bushel, however, is nearly 3 gallons. The theoretical yield of ethanol can be calculated from the stoichiometric relationships of starch, glucose, and ethanol by assuming 1.0 g of starch hydrolyzes to 1.11 g of glucose which then can be fermented to 0.511 g of ethanol (Thomas et al., 1996). Although ethanol plants approach the theoretical ethanol yield with some fermentation batches, the variability between batches is of concern and could be the primary benefit to developing hybrids for consistently high ethanol yields (Dr. Charlie Hurburgh, personal communication, 2007). Some of this unrealized potential is possibly due to shortcomings in current starch to ethanol conversion technology, but it is also likely a result of less than ideal physicochemical properties of starch that affect its digestibility. Understanding the characteristic differences between maize genotypes that are high and low in ethanol suitability, as well as how environmental stresses influence these differences will provide a rational basis for developing hybrids designed specifically for ethanol production,

rather than relying on the current practice of selecting hybrids from existing product offerings.

Variability in ethanol yield is well documented. Singh and Graeber (2005) examined the contributions of maize hybrid and environment to variation in ethanol yield. Environmental variability in ethanol yield was significant in one experiment using six hybrids produced at 4 locations. In a second experiment with 12 hybrids at four locations, however, environment was not a significant factor. They observed up to 22.7% variation in ethanol yield across hybrids and production environments when data from both experiments were combined. Their results indicate that a hybrid with the highest ethanol yield in one production environment may not perform similarly in another environment due to theorized differences in weather, soil, fertility, or other agronomic factors. Sharma et al. (2006a) utilized four commercial hybrids in a modified dry-grind process designed for hydrolysis of uncooked starch granules. Hybrid x environment interaction was noted for two of the four hybrids. Significant differences among hybrids were recorded for rate of fermentation and final ethanol concentration. Neither of these studies reported the initial starch content of the liquefied mash used for ethanol production. Therefore initial differences in kernel starch content due to genotype or environment may have contributed to some of the variability in ethanol yield. However, other studies (Murthy and Singh, 2005; Singh and Graeber, 2005) have indicated that little or no correlation exists between extractable starch and ethanol yield; it is unlikely that variability in starch content contributed to the observed differences in ethanol yield. This lack of correlation between starch content and ethanol yield has been noted in grain sorghum as well (Wu et al., 2006a). Proposed explanations for variation in ethanol yield are genetic and environmentally induced differences in starch structure, protein-starch matrices, and starch-lipid complexes; all of which could result in poor digestibility.

Starch granules vary considerably in their natural susceptibility to enzymatic digestion due to their structure (Fuwa et al., 1977; Kimura and Robyt, 1995). Fuwa et al. (1977), studying the susceptibility for various plant starches to amylase hydrolysis, reported that digestion susceptibility varied over 30-fold (0.89% to 29.4% glucose produced per hour). In normal starch granules, amylose content is concentrated at the exterior of the

granule where it forms tight associations with amylopectin. These associations decrease susceptibility to digestion, particularly in uncooked granules (Jane, 2006). Most plant starches contain about 20-25% amylose. But some, such as certain types of maize starch, contain up to 80% amylose. These high amylose types are considerably more resistant to hydrolysis. Additionally, the branch chain length of amylopectin influences granule crystallinity (Hizukuri, 1985). Granules with shorter amylopectin branch chains are less crystalline, and consequently, are hydrolyzed faster (Jane et al., 2003).

The high amylose content of maize and other grains has a negative effect on ethanol yield (Sharma et al., 2006b; Wu et al., 2006b). Wu et al. (2006b) reported the efficiency of starch conversion to ethanol decreased significantly when the amylose content of the starch exceeded 35%. In addition to the resistance to digestion previously reported by Fuwa et al. (1977), starches that are high in amylose typically have a higher gelatinization temperature due to increased hydrogen bonding between the linear molecules (Ellis et al., 1998). This is important in the dry-grind process because "cooking" is necessary to gelatinize the starch into a soluble form accessible to the hydrolyzing activity of α-amylase and glucoamylase.

These studies indicate that genetics and environment impact suitability of maize grain for ethanol production. Although much has been published on the relationship between starch structure and digestibility, this information has not been applied specifically to improve the efficiency of dry-grind ethanol production. Consequently, the physiological bases for the genotype x environment interactions on ethanol yield are largely unknown.

vi) Relationship between drought and mycotoxin accumulation

If water stress during grain filling affects starch accumulation and structure as suspected, it also may have an impact on the susceptibility of the grain to infection by certain pathogens and accumulation of mycotoxins. Aflatoxin produced by *Aspergillus flavus* and fumonisin produced by *Fusarium verticillioides* are two of the more common mycotoxins found in maize grain. Mycotoxins represent a serious threat to food safety and security worldwide. Aflatoxin is one of the strongest biological carcinogens (Windham and Williams, 1998). Fumonisin is responsible for equine leukoencephalomalacia (Wilson and Maronport, 1971) and porcine pulmonary edema (Harrison et al., 1990), both potentially fatal

diseases of livestock. Additionally, fumonisin B1 has been linked to esophageal cancer of humans (Munkvold and Desjardins, 1997). Clearly, there is great benefit to be realized from understanding the factors controlling mycotoxin accumulation in maize grain. This knowledge is prerequisite for developing sources of resistance or management practices to eliminate this problem.

Typically, the risk for *Aspergillus flavus* and *Fusarium verticillioides* infection and mycotoxin accumulation is greatest in production environments where drought conditions and high temperatures prevail (Betrán and Isakeit, 2004; White, 1999). Mycotoxins are prevalent in the southeastern United States for this reason. These conditions during grain filling, however, are not uncommon in Iowa as exemplified by drought conditions in eastern Iowa during 2005 which led to increased incidence of mycotoxin contaminated grain (Yang, 2005). In addition to climatic factors, evidence suggests that the composition of the grain influences the initiation of mycotoxin synthesis and its accumulation. Warfield and Gilchrist (1999) reported significant differences in fumonisin B1 production in kernels inoculated with *Fusarium verticillioides* at different developmental stages. Kernels inoculated at the blister stage supported less fumonisin accumulation than those at the dent stage. They concluded fumonisin accumulation was affected by substrate composition as well as by moisture content of the kernel. Bluhm and Woloshuk (2005) reported that the presence of amylopectin was conducive to fumonisin B1 accumulation. *Fusarium* did not produce high levels of fumonisin when grown on kernels of high amylose maize or an artificial medium containing amylose. But *Fusarium* produced high levels of fumonisin when grown on kernels of waxy maize (low amylose) or on artificial medium containing amylopectin. Such results imply that ease of starch digestibility could be an important factor affecting resistance to mycotoxin accumulation. In contrast to the study of Warfield and Gilchrist (1999), Bluhm and Woloshuk did not detect fumonisin production in blister stage kernels and only trace amounts in milk and dough-stage kernels. Although amylose and amylopectin were not examined specifically, studies utilizing *Aspergillus flavus* grown on media containing glucose, maltose, or maltotriose (fermentable sugars) initiated aflatoxin production while media containing more complex carbohydrates (i.e. starch) did not accumulate significant amounts of toxin (Woloshuk et al., 1997). Similarly, Fakhoury and Woloshuk (1999)

reported that an *Aspergillus flavus* mutant lacking α-amylase failed to produce aflatoxin when grown on a starchy medium.

Drought stress induced reduction of amylose accumulation or other modifications to starch digestibility might increase availability of simple carbohydrates for *Fusarium* or *Aspergillus*, and accumulation of mycotoxins. Since no major sources of resistance to mycotoxin accumulation are currently available, establishing a link between stress induced changes in starch digestibility and susceptibility to mycotoxin accumulation could be highly valuable. It would provide a rational basis for breeding efforts and management practices aimed at reducing the risk of aflatoxin or fumonisin production. It is important to realize, however, that developing maize genotypes specifically for increased digestibility for ethanol production could increase their vulnerability to mycotoxin contamination. This would present a major safety risk in terms of distillers dried grains with solubles (DDGS) for livestock feed. As demonstrated by Murthy et al. (2005), aflatoxin is resistant to the dry-grind ethanol process and passes through to the co-products in significantly high levels.

Experimental Hypotheses and General Approaches

The hypotheses of this thesis were:

1. Water stress during the grain filling influences kernel composition and starch structure. Changes in composition and structure are either a result of direct effects on storage product biosynthesis (transcriptional or metabolic regulation) or are simply a result of a shift in kernel development.
2. Altered composition, starch structure, or kernel physical properties as influenced by water stress result in variability in starch digestibility and potential for ethanol yield.
3. A shortened duration of grain filling reflects the decreased expression of key genes involved in starch and storage protein biosynthesis.
4. The shifts in kernel water content in water stressed kernels induce decreased transcription of those key genes.
5. Water stress during kernel development increases susceptibility to aflatoxin accumulation.

In line with these hypotheses, the approaches used were to i) grow maize kernels under defined water availability regimes, ii) measure kernel development patterns in each treatment, iii) analyze the composition and starch digestibility in each treatment, iv) measure the expression of key genes in starch and protein synthesis, and v) assess the potential for aflatoxin accumulation in irrigated and water stressed maize kernels. While both fumonisin and aflatoxin accumulation in maize kernels have been linked to carbohydrate availability and fungal α-amylase activity, aflatoxin was chosen for this study due to its more consistent association with drought stress conditions.

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Table 1. Grain composition of maize inbred lines grown in Ames, IA during summer 2006. Measured using near-infrared spectroscopy (NIR).

Inbred	Type	% Starch†	% Protein	% Oil
A451	Floury/dent	67.5	15.6	3.6
B73	Semident	69.7	12.5	3.8
B84	Dent	70.4	12.0	3.6
B97	Dent	70.2	11.4	4.0
B100	Semident	69.2	11.9	4.6
B104	Dent/semident	70.6	12.0	3.6
B114	Dent	70.0	11.3	4.2
B116	Dent	70.0	12.5	3.5
B117	Dent	69.5	12.2	4.0
B122	Dent	69.1	12.6	4.0
B123	Dent	67.2	14.3	4.8
B125	Dent	69.8	11.9	3.7
B126	Dent	69.1	13.6	3.4
B129	Dent	69.5	13.1	3.5
B130	Dent	69.7	12.0	3.9
B131	Dent	68.0	13.6	3.9
H22X	Dent	68.1	11.8	5.9
IDS91	Pop	69.8	12.6	3.4
Mo46	Semi-flint	69.3	12.6	4.0
N209	Dent	67.9	12.7	4.7
Pa760	Dent	68.2	14.5	3.6
	Minimum	67.2	11.3	3.4
	Maximum	70.6	15.6	5.9
	Mean	69.2	12.7	4.0

† Percentages shown have been corrected to 0% moisture content basis.

Chapter 2. Maize Kernel Development, Composition, and Starch Digestibility as Affected by Water Stress during Grain Filling

A manuscript to be submitted to Crop Science

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Abstract

Environmental stress during grain filling in maize (*Zea mays* L.) can have a dramatic effect on kernel development and yield. The influence of environmental perturbations such as water stress on grain composition, however, is not as well documented. The objective of this research was to examine the impact of water stress during grain filling on kernel growth, resulting grain composition, and susceptibility of kernel starch to hydrolysis. Efficient starch hydrolysis is of great importance to the dry-grind ethanol industry and understanding the impact of environment on grain quality is central to developing maize germplasm suitable for ethanol conversion efficiency as well as to managing variation in ethanol production. A terminal water stress imposed at 17 days after pollination decreased final kernel dry weight by 21.2% ($P < 0.0001$), kernel specific density by 1.9% ($P < 0.05$), and mean starch granule diameter by 9% ($P = 0.053$) relative to the irrigated treatment. Starch, protein, and oil contents (milligrams per kernel) all were reduced with the decrease in kernel dry weight. The concentrations of these components, however, were not significantly affected. Solubilized starch from water stressed kernels was more susceptible to hydrolysis by glucoamylase ($P < 0.0001$) and bacterial α-amylase ($P < 0.0001$). This increased susceptibility to hydrolysis likely resulted from a lesser kernel density and a greater proportion of fine particles in the milled kernels of water stressed plants. These results suggest that water stress during grain filling alters the composition and physical properties of maize kernels in a way that favors ethanol conversion efficiency. Use of maize grain for ethanol production, however, would be negatively affected by the reduction in total starch accumulation.

Introduction

Maize (*Zea mays* L.) production is becoming increasingly focused on key end-use markets that have specific, divergent criteria for grain quality traits. In the case of the dry-grind ethanol industry, grain that contains more highly digestible starch is desired to improve both processing efficiency and ethanol yield per unit weight of grain. Breeding and transgenic approaches to improve maize grain for ethanol production are underway within the seed industry (Bothast and Schlicher, 2005; Singh et al., 2006). Variation in kernel composition and starch digestibility resulting from environmental stresses such as limited water availability, however, may impede the progress of these germplasm characterization and improvement efforts.

Grain composition and the resulting physicochemical properties of grain are determined during seed development. As such, perturbations to seed development might also influence grain composition. Reproductive development in maize is sensitive to plant water stress during anthesis, early seed development, and grain filling (Saini and Westgate, 2000; Boyer and Westgate, 2004). Although the early stages of maize reproductive development are the most vulnerable to water deficit and are likely to result in the greatest total yield reduction, the grain filling period is also adversely affected. For example, Westgate and Boyer (1985) subjected maize plants to controlled water stress treatments at three points during seed development. Stress applied at anthesis resulted in complete kernel abortion while stress applied at mid-grain fill resulted in a 36% reduction in seed dry weight relative to the irrigated control. Westgate and Thomson Grant (1989) performed a similar experiment in which they subjected maize plants to transient water stresses. When stressed at mid-grain fill for a period of six days, final kernel dry weight accumulation was unaffected. Taken together, these studies clearly indicate that the timing and duration of a water stress mediate the magnitude of any negative affect on kernel development.

Water stress imposed after sink capacity (i.e. endosperm cell number) has been established primarily affects the duration of the effective period of grain filling with little change in the rate of dry matter accumulation (Westgate, 1994). Clearly, a shortened period of dry matter accumulation limits the total amounts of starch, protein, oil, and other seed constituents that are stored in either the endosperm or embryo. These components of seed

composition have temporal patterns to their accumulation (Ingle, 1965). For example, starch is synthesized throughout grain filling (Zhang et al., 2008), while zeins, the major class of endosperm storage protein, accumulate primarily during the later stages of seed development (Monjardino et al., 2005). Starch structure also changes throughout maize endosperm development. Amylose concentration increases gradually while amylopectin branch chain synthesis displays different phases of elongation (Li et al., 2007). As such, a shortened grain filling period due to water stress might alter the chemical composition as well as the physical characteristics of the kernel.

There are few reports of the influence of water stress on maize seed composition. Jurgens et al. (1978) maintained stressed maize plants at a constant water potential. The stressed plants produced kernels with increased protein concentration (+24%) and decreased oil concentration (-18.4%) relative to the irrigated control plants. These results are supported by those of Harrigan et al. (2007). In a survey of seven maize hybrids grown under irrigated and water stressed regimes, oil and starch concentrations were reduced while protein concentration was increased by drought. Clearly, variation in seed composition due to water stress related perturbations to seed development is likely to affect the chemical and physical properties of the grain. But there is little information available on how this variation might affect industrial uses of maize grain that have specific quality requirements such as the dry-grind ethanol industry.

Production of fuel ethanol from maize grain has rapidly increased in output, particularly in the central region of the United States. In 2007, 6.5 billion gallons of ethanol were produced in the United States (RFA, 2008). Most of this ethanol was produced from maize utilizing more than 3.2 billion bushels of grain (NCGA, 2008). In response to this growing industry, companies that develop maize germplasm are promoting hybrids with characteristics especially suitable for dry-grind ethanol production. Characterization studies of maize hybrids have revealed variation in ethanol yield attributable to both genetic and environmental causes. For example, Singh and Graeber (2005) reported the ethanol yields of eighteen yellow dent corn hybrids grown in different locations. Although environment was significant in only one of two experiments, considerable variation was measured among hybrids. Variation of 22.7% in ethanol yield was observed among hybrids across different

locations. Similarly, Sharma et al. (2006) reported genotypic and environmental variation for rate of fermentation, final ethanol concentration, yeast glucose uptake, and yields of co-products in four different maize hybrids. Differences in grain physical or chemical properties underlying the observed variation in these studies were not identified. As such, further breeding efforts to improve maize hybrids for ethanol production must understand the mechanisms underlying variation to increase the efficiency of germplasm improvement.

In the dry-grind ethanol process, α-amylase and glucoamylase are used to hydrolyze starch to fermentable glucose. Solubilization of the starch and initial hydrolysis are accomplished by high temperatures and the accompanying use of thermostable α-amylase. Glucoamylase completes the hydrolysis of starch to glucose. Therefore, variation in ethanol production attributable to feedstock characteristics is likely to result from incomplete hydrolysis of starch to glucose. For example, Dien et al. (2002) compared five maize hybrids for ethanol conversion efficiency using a conventional dry-grind ethanol process. Although the hybrids possessed similar grain starch concentrations, ethanol conversion efficiencies varied from 87 to 96%, indicating that some characteristic of the grain limited starch hydrolysis. A number of factors are thought to influence the susceptibility of starch to enzymatic hydrolysis in human, animal, and industrial systems. These factors include the ratio of amylose to amylopectin within starch, inaccessibility of the starch in large particles, protein-starch binding, and susceptibility of the raw starch granule to enzymatic attack. Conventional dry-grind production of ethanol is unique in that starch is almost completely solubilized by jet-cooking so susceptibility of the raw starch granule to enzymatic hydrolysis may be disregarded as a limiting factor.

Starch is composed of amylose and amylopectin molecules; both are homopolymers of glucose. Typically, normal maize starch contains 25-30% amylose and 70-75% amylopectin on a dry-weight basis (Boyer and Shannon, 2003). The ratio of these two molecules along with properties specific to each influences the physicochemical characteristics of starch including susceptibility to enzymatic hydrolysis. For example, amylose molecules form complexes with lipids; these complexes limit access of starch hydrolytic enzymes to amylose (Seneviratne and Biliaderis, 1991). The ethanol conversion efficiencies of starches varying in amylose concentration have been reported (Sharma et al.,

2007; Wu et al., 2006). Both studies indicate that although there is a negative relationship between amylose concentration and starch hydrolysis, ethanol yield is not significantly affected until amylose concentration exceeds 35% of total starch. Although the range of amylose concentration in normal maize genotypes is less than 35%, the response of amylose concentration in maize to environmental stress is largely unknown. In a study of two maize genotypes grown under optimal (25^{o}C) and high (35^{o}C) temperatures, both apparent and true amylose concentrations were decreased by high temperature during grain fill (Lu et al., 1996). A similar response might be possible in response to water stress although it is not clear if a small change in amylose accumulation might affect susceptibility of the starch to enzymatic hydrolysis.

Starch granules within the maize endosperm exist in a matrix of zein bodies. The protein matrix that contains starch granules has also been proposed as a potential factor limiting starch hydrolysis and ethanol conversion efficiency. Wu et al. (2006) found no significant effect of protein concentration on ethanol production. Similarly, grain protein concentrations of 5.3-12.7% did not have a significant effect on starch hydrolysis rate (Uppalanchi, 2005). The types of storage proteins (e.g. zeins) that accumulate in the endosperm, however, can vary with genotype, environmental conditions, and agronomic practices (Hamilton et al., 1951; Holding and Larkins, 2006). These different forms of zein fulfill different roles in the endosperm influencing hardness, grinding characteristics, and susceptibility to fungal infection of the kernel (Chandrashekar and Mazhar, 1999). Hardness and grinding characteristics might be of particular importance as the particle size distribution of ground maize grain could also influence the amount of glucose liberated during starch hydrolysis. Ethanol yield is greatest when a screen with small openings is used during grinding (Naidu et al., 2007). The small particles that result have a large surface area to mass ratio which facilitates higher enzymatic reaction rates. Softer, lower density kernels might produce a particle size distribution consisting of a greater proportion of fine particles. Therefore, the types of proteins being synthesized and resulting hardness of the grain might be a more important factor than total protein content for starch hydrolysis.

A more detailed understanding of environmental effects on maize grain composition, amylose content, and other kernel traits is needed to establish the bases for variability in

susceptibility to enzymatic hydrolysis and potential for resulting ethanol yield. The objective of the present study was to investigate the relationship between altered kernel growth, grain composition, and susceptibility of kernel starch to enzymatic hydrolysis using water stress as a perturbation to development.

Materials and Methods

Experimental Design

Two treatments were included in the study; irrigated and water stressed. Plants were blocked by treatment due to the necessity to control water availability. Within each block, plants were designated for specific sampling dates which were randomized to minimize positional effects. Three replicates per treatment were designated for each sampling date prior to physiological maturity. Five replicates per treatment were sampled at physiological maturity. The experiment was repeated two times and data for common sampling dates were pooled.

Greenhouse Plant Care

Maize plants (inbred B73) were grown in the Iowa State University Department of Agronomy greenhouses in individual 19 L plastic pots containing a commercially available potting mix (Sunshine SB300 Universal, SunGro Horticultural, Bellevue, WA). Plant density was 2.4 plants m^{-2}. Growth conditions consisted of a 15-h photoperiod and $27^{o}C/18^{o}C$ maximum/minimum temperatures. Fertilizer (15-5-15; N-P-K) was injected into the irrigation water at a volumetric ratio of 1:40. After pollination, irrigation was managed automatically by GP1 data loggers, and SM200 soil moisture sensors (Delta-T Devices, Cambridge, UK) indicated the range of soil moisture content values for irrigated and water stress treatments. Water was withheld from the water stress treatment beginning at 17 days after pollination (DAP) and continued until physiological maturity. Fertilizer was withheld from both treatments after 17 DAP to minimize effects due to differences in nutrient availability.

Soil Moisture Content and Leaf Water Potential

Volumetric soil moisture content (m^3 water m^{-3} soil) was measured by the data loggers and soil moisture sensors previously described. Soil moisture content is not necessarily representative of plant water status. As such, mid-day leaf water potential was measured during mid-grain fill using isopiestic thermocouple psychrometry corrected for heat of respiration (Boyer, 1995). Briefly, leaf squares (green tissue only) were excised from the leaf subtending the ear and immediately sealed in brass psychrometry cups. The samples were placed in a 26°C water bath and a dry thermocouple reading was obtained to allow for correction for heat of respiration. Sucrose solutions of known osmotic potential were applied to the thermocouple ring until a reading near zero was obtained. Water potentials (ψ_w) were calculated as described by Boyer (1995).

Statistical Analysis

Leaf water potential means were compared for the two treatments using a two-sample t-test and the assumption of equal variance (PROC TTEST; SAS 9.1, SAS Institute Inc., Cary, NC).

Kernel Development

The plants were self- or sib-pollinated and ears were harvested at intervals from 12 to 40 DAP. Ears from each treatment harvested at each sampling date were placed in air-tight plastic bags for transport to the laboratory. Fifteen kernels from the middle one-third of the rachis were removed in a humidified box maintained at saturation vapor pressure and used for dry weight (DW), fresh weight (FW), water content (WC), and % moisture content (% MC) measurements. Dry weights were obtained after drying the kernels to constant weight at 65°C. Percent moisture content (fresh basis) was calculated according to Equation 1.

$$\% \text{ MC} = \left(\frac{\text{FW} - \text{DW}}{\text{FW}} \right) \times 100 \qquad (1)$$

Statistical Analyses

Percent moisture content was linearly regressed versus days after pollination for each treatment. Slopes and intercepts were compared using appropriate t-tests. A bi-linear with plateau model (Equations 2 and 3) was fitted iteratively for dry weight versus % MC to compare rates and durations of dry matter accumulation between the two treatments (TableCurve 2D v5.01, Systat Software Inc., San Jose, CA).

$$y = a + b(100 - \%MC), \qquad \text{for } \%\ MC \geq c \tag{2}$$

$$y = a + b(100 - c), \qquad \text{for } \%\ MC < c \tag{3}$$

In these equations *a* is the y-intercept and *b* is the slope of the line at % MC values greater than *c*. The variable *c* is the breakpoint at which maximum dry matter has been accumulated.

Grain Composition and Density

Total starch, oil, and protein concentrations in mature kernels were analyzed by near-infrared spectroscopy (NIR; Iowa State University Grain Quality Lab). Specific density (g cm^{-3}) was measured with an Accupyc 1330 nitrogen gas pycnometer fitted with a 35 cm^3 chamber (Micromeritics, Atlanta, GA). Starch concentration in excised, lyophilized endosperm and embryo tissue was determined following three extractions of soluble sugars with 800 ml L^{-1} ethanol at 60°C. The starch contained within the tissue was hydrolyzed with glucoamylase from *Aspergillus niger* (Sigma A7420; 0.8 units per mg of tissue) at 60°C for 12 h. Following hydrolysis, glucose was quantified spectrophotometrically at 540 nm (AACC Method 76-11). Starch was calculated as 90% of glucose liberated. Total nitrogen content in excised, lyophilized endosperm and embryo tissue was determined by combustion analysis (Iowa State University Soil and Plant Analysis Laboratory). Total nitrogen was converted to protein using a conversion factor of 6.25.

Statistical Analyses

Grain composition and density means for the two treatments were compared using two-sample t-tests with the assumption of equal variance (PROC TTEST; SAS 9.1, SAS Institute Inc., Cary, NC).

Starch Isolation

Starch from mature maize kernels was isolated using a small-scale wet milling method (White et al., 1990). Kernels were soaked in 24 mmol L^{-1} $Na_2S_2O_5$ for 48 h at 50°C. Pericarp and embryo tissues were removed manually. Endosperm tissue was homogenized in absolute ethanol for 3 min. The resulting slurry was passed over a 53 μm screen. Fiber trapped on top of the screen was again homogenized with absolute ethanol and passed over the screen. The filtrate was centrifuged at 6900 x *g* for 15 min. The supernatant was discarded and the starch pellet resuspended in a 0.1 mol L^{-1} NaCl solution containing 100 ml L^{-1} toluene to remove proteins. This mixture was stirred for one hour. The toluene layer was discarded and washing was repeated until the toluene layer became clear. Finally, the starch pellet was washed twice with deionized water and once with absolute ethanol followed by drying at 30°C.

Potentiometric Iodine Titration for Apparent Amylose Content

Apparent amylose content of starch in mature kernels was measured using the potentiometric iodine titration method of Schoch (1964). This method may overestimate amylose content due to the presence of long-branch chains of amylopectin, but remains an effective approximation technique. Three biological replicates (grain bearing ears) from each treatment were selected for analysis and starch was extracted from mid-rachis kernels as described previously. Briefly, starch was dispersed and defatted in 900 ml L^{-1} dimethyl sulfoxide at 100°C (Lim et al., 1994). The defatted starch was precipitated using absolute ethanol and dried at 30°C. The starch precipitate (100 mg) was redissolved in water with gentle heating. Potassium iodide (0.5 mol L^{-1}) was added to the sample prior to titration. The titrant consisted of 0.5 mol L^{-1} KI, 0.5 mol L^{-1} KCl, and 2 mg ml^{-1} I_2 added in 2 ml increments by a 702 SM Titrino autotitrator (Metrohm AG, Switzerland). Bound iodine was

plotted versus free iodine and the iodine affinity (Equation 4) of the sample calculated by extrapolating the upper, linear portion of the curve to the y-axis. Percent apparent amylose content was calculated by dividing iodine affinity by 19.0, the approximate iodine affinity of purified maize amylose (Equation 5).

$$\% \text{ Iodine affinity} = \left(\frac{\text{mg bound iodine at zero intercept}}{\text{mg sample weight (dry basis)}} \right) \times 100 \quad (4)$$

$$\% \text{ Apparent amylose content} = \left(\frac{\% \text{ iodine affinity}}{19.0} \right) \times 100 \quad (5)$$

Statistical Analysis

Apparent amylose content means were compared for the two treatments using a two-sample t-test and the assumption of equal variance (PROC TTEST; SAS 9.1, SAS Institute Inc., Cary, NC).

Starch Granule Size Measurements

Endosperm starch was isolated as described above for three replicates from each treatment. Starch granules were mounted on the surface of a brass disk using double-sided adhesive silver tape. Granules were coated with gold/palladium (60/40) and imaged under a scanning electron microscope (JEOL model 5800LV; JEOL Ltd., Tokyo, Japan) at 500x and 1200x magnification at the Iowa State University Microscopy and NanoImaging Facility. The images obtained at 500x were used for making granule size measurements. The internal scaling feature of the image analysis software was calibrated to measure in μm. Diameter measurements from the horizontal axes of granules from three separate images for each sample were obtained. At least 400 granules from each replicate were measured.

Statistical Analysis

Mean granule diameters were compared for the two treatments using a two-sample t-test and the assumption of equal variance (PROC TTEST; SAS 9.1, SAS Institute Inc., Cary, NC).

Particle Size Distribution Analysis

Grain samples from three ears of each treatment were ground for one minute using a Stein Laboratory Mill (Model M2; The Steinlite Corporation, Atchison, KS). The resulting corn flour was analyzed for particle size distribution using an Advantech Sonic Sifter equipped with 18, 35, 60, and 100 mesh screens (Advantech Manufacturing, New Berlin, WI). Samples of 1 g (dry weight) were sifted for two minutes at amplitude 6.

Statistical Analysis

Particle size distribution means were compared for the two treatments using a two-sample t-test and the assumption of equal variance (PROC TTEST; SAS 9.1, SAS Institute Inc., Cary, NC).

Starch Hydrolysis

Grain samples were ground as previously described. Three biological replicates (grain bearing ears) were selected from each treatment for the starch hydrolysis experiments. Glucoamylase and α-amylase assays were used to determine the susceptibility of kernel starch to enzymatic hydrolysis.

Susceptibility to hydrolysis by glucoamylase

Small samples (5 mg fresh weight) of corn flour were transferred to 0.2 ml PCR (polymerase chain reaction) tubes. Sodium acetate buffer (50 mmol L^{-1} NaOAc, 5 mmol L^{-1} $CaCl_2$, adjusted to pH 4.5) was added to each tube at a volume of 100 µl. The tubes were placed in a PCR thermocycler (MJ Research PTC-200 DNA Engine; Bio-Rad Laboratories, Hercules, CA) and the starch gelatinized at 95^{o}C for 10 min. After gelatinization, 4 µl of glucoamylase (240 AGU g^{-1} of corn flour) from *Aspergillus niger* (Sigma A7420) were added to each tube. The tubes were returned to the thermocycler and incubated at 60^{o}C. Tubes were removed at 0, 0.5, 1, 2, 3, 4, and 5 h for analysis. Glucose was quantified spectrophotometrically as previously described and expressed as percent hydrolysis of the total starch estimated to be in the sample. Total starch estimates were based on concentration of starch as previously measured by NIR and the dry weight of each sample. The experiment was replicated three times.

Susceptibility to hydrolysis by α-amylase with and without protease pre-treatment

The method of Pérez-Carillo and Serna-Saldívar (2007) was followed with modifications. A 2 x 2 x 3 factorial experiment was designed in which irrigation treatment (water stress vs. irrigated), protease treatment (with protease vs. without protease), and particle size (unsieved, coarse, and fine) were factors. One gram (fresh weight) of corn flour was transferred to flasks containing 20 ml of 0.1 mol L^{-1} phosphate buffer adjusted to pH 7.0. Coarse (≥ 60 mesh) and fine (< 60 mesh) particles were obtained by grinding kernels as previously described and passing the resulting flour over a 60 mesh screen. The flasks were placed in a 60°C shaking water bath and brought to temperature after which 5.0 µl of Neutrase 0.8L (protease from *Bacillus amyloliquefaciens*; Novozymes, Bagsvaerd, Denmark) were added to the flasks designated for protease pre-treatment. The samples were incubated for 2 h. Following protease pre-treatment, all flasks received 10.4 µl (240 U g^{-1} of corn flour) of Termamyl 120L (Novozymes, Bagsvaerd, Denmark), a thermostable α-amylase from *Bacillus licheniformus*. The temperature of the water bath was increased to 90°C over a period of approximately 50 min. Aliquots were removed from the flasks at 0, 1, 2.5, and 4 h and analyzed for reducing sugars and total carbohydrates. Reducing sugars were measured using the method of Miller (1959) and expressed as glucose equivalents. The phenol-sulfuric acid method of total carbohydrate analysis (this method is not specific to a particular carbohydrate) was used with glucose as a standard (Dubois et al., 1956). The experiment was replicated three times.

A similar approach was used for determining total hydrolysis susceptibility. After 2.5 h of α-amylase treatment, the pH of the buffer solution was adjusted to pH 4.5. All flasks received 8.5 µl (3.4 AGU g^{-1} of corn flour) of AMG 300L (Novozymes, Bagsvaerd, Denmark), a glucoamylase from *Aspergillus niger*. The flasks were incubated for 24 h at 60°C. Reducing sugars and total carbohydrates were assayed as previously described.

Statistical Analyses

The non-linear model (Equation 6) of Goñi et al. (1997) was used for the glucoamylase susceptibility results.

$$\% \text{ hydrolysis} = C\left(1 - e^{(-kt)}\right) \quad (6)$$

In this equation *C* is the final % hydrolysis obtained, *k* is the rate constant with units of reciprocal time, and *t* is time. The PROC NLIN routine was used to fit the model to each treatment (SAS 9.1; SAS Institute Inc., Cary, NC). A sum of squares reduction test was used to assess treatment differences. The results from the α-amylase and protease susceptibility experiment were analyzed with PROC GLM (SAS 9.1; SAS Institute Inc., Cary, NC).

Results and Discussion

Soil water restriction was imposed at 17 DAP and drying progressed steadily with time (Figure 1). The greatest rate of soil water drying occurred during the first five days of the treatment. During this period the rate of drying was 0.038 m^3 d^{-1}. Additional drying occurred until harvest of the physiologically mature samples (~ 40 DAP) at which point the volumetric soil moisture content was less than 0.1 m^3 m^{-3} soil.

Leaf water potential measurements taken during the active grain filling period revealed a significant difference between the irrigation treatments. The mean leaf water potential for the irrigated treatment was -1.09 MPa, while the mean leaf water potential for the water stress treatment was -2.08 MPa (Figure 2). A t-test of the difference in mean leaf water potentials was highly significant ($P < 0.0001$). The water potential for the irrigated treatment was less than those reported by Westgate and Thomson Grant (1989) for irrigated maize plants; however, it is still an acceptable value for well-watered, actively growing maize plants. Boyer (1970) reported that maize photosynthesis is inhibited at low leaf water potentials. As such, photosynthesis in the water stressed plants was expected to be inhibited, although photosynthetic parameters were not measured. Additionally, green leaf area in the water stressed treatment was less than 50% by 5 days after the beginning of the treatment and less than 10% by 10 days after the beginning of the treatment (data not shown). Therefore, assimilation of carbon for most of kernel development in the water stressed treatment was likely from remobilization of vegetative reserves rather than from current photosynthesis.

The negative linear relationships between percent moisture content on a fresh basis and days after pollination were not significantly different ($P = 0.163$) for the two treatments

based on a t-test of the regression slopes (Figure 3). As such, treatment differences could be normalized on a percent kernel moisture content basis according to Borrás and Westgate (2006). Fresh weight increased rapidly in both treatments reaching a maximum of about 340 mg per seed for the water stress treatment and approximately 397 mg per seed for the irrigated treatment (Figure 4A). These maximum fresh weight values corresponded to % MC values of 47% for the water stress treatment and 43% for the irrigated treatment. After reaching maximum values, fresh weight declined in both treatments as water content was replaced by dry matter accumulation and acquisition of desiccation tolerance was initiated. Water content values were not significantly different between treatments through 17 days after pollination (Figure 4C). By 25 days after pollination, the mean water content in the water stress treatment appeared to be less than that of the irrigated treatment and did not reach the same maximum value. Dry matter accumulation (Figure 4B) proceeded in a similar fashion in both treatments. A bi-linear with plateau model describing rate and duration of dry matter accumulation was fit for each treatment (Figure 5). It is important to note that the limited number of sampling points at or past physiological maturity is likely to affect confidence in the values predicted for transition from linear grain filling to maximum dry weight accumulation. A t-test comparison of slopes indicates that the rate of dry matter accumulation was not significantly different between the treatments ($P = 0.176$). Rather, the duration of grain filling was shortened in the water stress treatment (Figure 5). Dry matter accumulation in the water stress treatment ended at approximately 48% moisture content while it ended at approximately 40% in the irrigated treatment ($P = 0.007$). The effective period of grain filling in the water stress treatment was shortened by approximately 5 days relative to the irrigated treatment.

At physiological maturity, mean kernel dry weight in the water stress treatment was 188 mg per kernel while the mean dry weight of irrigated kernels was 238 mg per kernel (Table 1). This represents a 21.2% reduction in kernel dry weight relative to the irrigated kernels ($P < 0.0001$). In addition to reduced dry weight, water stressed kernels were less dense ($P < 0.05$). Taken together, these results suggest that the synthesis of primary storage reserves (e.g. starch and protein) was limited by the water stress. Additionally, a greater

proportion of kernels from the water stress treatment had void spaces in the central endosperm which partially explains their lower density (visual assessment; data not shown).

Although kernel dry weight was reduced dramatically by water stress, changes in kernel composition were minor (Table 2). Total protein concentration was 12.9% in the water stress treatment and 12.1% in the irrigated treatment; however, the treatment means were not significantly different (P = 0.206). Starch concentration in the water stressed kernels was not significantly different (P = 0.599) from that observed in well watered kernels. Water stressed kernels had an endosperm starch concentration of 80.2% while irrigated endosperms had a starch concentration of 81.4% although the means were not significantly different (P = 0.645). Embryo starch concentration in water stressed kernels was 6.3% compared to 5.5% in irrigated kernels (P = 0.178). Although not significant, the possible increase in endosperm protein concentration and decrease in endosperm starch concentration under water stress conditions are consistent with the results of Jurgens et al. (1978) and Harrigan et al. (2007).

The water stress treatment did not significantly affect embryo weight (P=0.231); embryo weight averaged across the two treatments was 20.2 mg. Yet water stressed kernels had a significantly greater ratio of embryo to kernel dry weight (0.105) compared to irrigated kernels (0.087; P = 0.012). The maintenance of embryo growth under water stress conditions suggests that the embryo has priority for assimilate uptake relative to the endosperm tissue. One possible mechanism for this might be the high affinity of the embryo sugar absorption system proposed by Griffith et al. (1987).

The increased embryo to kernel weight ratio in the water stress treatment stemming from perturbed endosperm development suggests that oil concentration would increase as a result of a greater proportion of embryo tissue, yet this did not occur. Total kernel oil concentration in water stressed kernels (3.9%) was similar to that of irrigated kernels (4.1%; P = 0.064). Therefore, it is likely that the metabolism of oil accumulation in the embryo was directly affected by water stress. Although the oil concentration of isolated embryos was not measured, the hypothesis that embryo oil accumulation decreased as a result of water stress can be supported by calculation. Approximately 83% of total kernel oil content accumulates in the embryo while the embryo is composed of approximately 33% oil (Watson, 2003).

Using the kernel oil concentrations and kernel dry weights measured in this study (Tables 1 and 2), the average kernel oil content in the water stress treatment is 7.3 mg kernel^{-1} while the average oil content of irrigated kernels is 9.8 mg kernel^{-1}. If it is assumed that 83% of this oil is in the embryo, the predicted oil content of water stressed embryos is approximately 6.1 mg and the oil content of irrigated embryos is approximately 8.1 mg. Using the average embryo dry weight of 20.2 mg, the predicted oil concentration of water stressed embryo tissue is approximately 30% while the oil concentration of irrigated embryos is about 40%. This theoretical approach indicates that embryo oil concentration decreased by about 25% in water stressed kernels relative to irrigated kernels; however, the difference is not apparent by comparing oil concentration on a total kernel weight basis due to the greater proportion of embryo tissue in water stressed kernels. Further work should examine the affect of water stress on embryo oil accumulation to support this hypothesis.

The lack of significance for several kernel composition traits underscores the challenges of working with limited replications of single ear samples. For example, single ears are subject to experimental errors associated with variation in kernel number. In this experiment, ears in the irrigated treatment had on average 90 fewer kernels per ear than ears in the water stress treatment ($P = 0.02$). This is not likely to be associated with a biological process but rather due to human error and fewer replications in the water stress treatment. Ears with fewer kernels are likely to experience a higher source to sink ratio which might influence the responses of starch, protein, and oil accumulation in different ways. For example, Borrás et al. (2002) showed that protein was more limited than starch at lower source-sink ratios. Despite differences in kernel number, the primary developmental difference between the treatments appeared to be the duration of dry matter accumulation. Borrás et al. (2003) reported no change in grain filling duration when kernel number was reduced through restricted pollination. Kernels from ears subjected to restricted pollination were larger and grew at an increased rate relative to the naturally pollinated ears. In the present study, however, the rate of dry matter accumulation between treatments was not significantly different ($P = 0.176$) so differences in kernel weight can be attributed primarily to the duration of grain filling.

When starch was expressed on a content basis (mg kernel^{-1}), there was a positive linear relationship between kernel weight and starch content (Figure 6A). A t-test comparison of the regression slopes was significant at P = 0.09. On a dry-weight basis, starch comprises 68-74% of total kernel weight (Watson, 2003). As such, a reduction in kernel weight is likely to be accompanied by a concomitant decrease in starch content, in agreement with our results. Similarly, protein and oil contents of water stressed kernels were less than those of irrigated kernels (Figures 6B and 6C).

Apparent amylose content was not significantly different between water stressed and irrigated kernels (P = 0.636). The mean apparent amylose content (% of total starch) for the two treatments was 25.4%. This value is consistent with the absolute amylose content of 24.4% reported for B73 by Li et al. (2006). The data of these authors also suggest that amylose accumulation for B73 endosperm starch reaches its maximum by 30 days after pollination. Therefore, a reduction in the duration of grain filling with little change in rate of starch accumulation is unlikely to perturb amylose synthesis in a way that would affect grain physicochemical properties.

Concentrations of reducing sugars and total soluble carbohydrates were higher in mature, irrigated kernels relative to water stressed kernels (Table 2). Reducing sugars in water stressed kernels decreased to 6.1 mg g^{-1} dry weight from 9.4 mg g^{-1} dry weight in irrigated kernels (P < 0.0001). Total soluble carbohydrates measured as glucose equivalents were 33.5 mg g^{-1} in water stressed kernels and 37.3 mg g^{-1} in irrigated kernels (P = 0.023). These results indicate that kernels from neither treatment stopped growing due to total depletion of carbohydrate reserves; however, the sugar concentration at which kernel development becomes impaired is not well characterized. Also, it is important to note that steady-state concentrations of carbohydrates are not representative of the flux of these compounds into the developing kernel. It is possible that water stressed kernels had a reduced influx of assimilates to the kernel which might limit kernel growth.

Water stress decreased mean starch granule diameter. On average, water stressed granules had a diameter of 10.1 μm while granules from irrigated kernels had a diameter of 11.1 μm (Table 1; P = 0.053). Granule diameter ranged from 9.3 to 10.9 μm in water stressed kernels and 10.9 to 11.4 μm in irrigated kernels. Starch granules grow by apposition

(elongation of existing starch molecule chains). Therefore, a shortened duration of grain filling which limits total starch accumulation and kernel dry weight is likely to do so by limiting granule expansion. Although starch granules are gelatinized during conventional dry-grind ethanol production, alternative methods have been developed which utilize specialized enzymes for hydrolyzing granular starch (Wang et al., 2007). In processes such as these, small granules might be more advantageous as they are hydrolyzed at a greater rate relative to large granules (Knutson et al., 1982; Kong et al., 2003). Other starch characteristics which are influenced by starch granule size include thermal and pasting properties (Ji et al., 2003; Narváez-González et al., 2007). For example, Ji et al. (2003) reported that onset gelatinization temperature was positively and significantly correlated with granules >13 μm in diameter and negatively and significantly correlated with granules 5-9 μm in diameter. Dry-grind ethanol production requires a large input of thermal energy to disrupt granular structure prior to enzymatic hydrolysis so small granules might be beneficial to the overall energy balance of the process.

Particle size distributions of ground corn flour from each treatment suggest that drought stressed kernels result in a greater proportion of fine particles less than 250 μm in diameter. When individual screen size means were compared by a t-test, irrigated kernels produced a larger proportion of $500 \leq x < 1000$ μm particles than did water stressed kernels ($P = 0.022$). Water stressed kernels produced a larger proportion of kernels in the $150 \leq x < 250$ μm diameter range ($P = 0.076$). Treatment comparisons for all other screen sizes were not significant ($P < 0.1$). When coarse particles (≥ 250 μm) and fine particles (< 250 μm) were summed for each treatment, both comparisons were significant. Irrigated kernels resulted in 64% coarse particles versus 57% coarse particles for water stressed kernels ($P = 0.029$). Therefore, irrigated kernels generated a smaller proportion (36%) of fine particles than water stressed kernels (43%; $P = 0.033$). The decreased density of water stressed kernels likely resulted in the greater susceptibility to milling as indicated by the greater proportion of smaller particles. Water stressed kernels also appeared to have less vitreous or hard endosperm relative to irrigated kernels. Although not measured by this study, it is possible that water stress impaired the synthesis of certain storage proteins or the assembly of protein

bodies within the endosperm. For example, reduced α-zein concentration has been correlated with increased susceptibility to milling processes (Mestres and Matencio, 1996).

We tested whether the different distribution of particle sizes would lead to a difference in susceptibility to starch hydrolysis and potential for ethanol yield. Ground corn flour from water stressed kernels exhibited a greater extent of hydrolysis by glucoamylase, as predicted by non-linear regression of Equation 6. Flour from water stressed kernels was 17.8% more susceptible to glucoamylase hydrolysis than was flour from irrigated kernels (Figure 8; $P < 0.0001$). Although still less, the % hydrolysis of flour from irrigated kernels was not significantly different from that of water stressed kernels after five hours ($P = 0.333$). These results suggest that total starch hydrolysis might not be affected despite an apparent increase in the initial rate of hydrolysis. Isolated starch from each treatment was compared using the same method as that for ground corn flour. There was no difference in hydrolysis characteristics between treatments for isolated starch granules (data not shown). This result implies that a factor other than starch structure was responsible for the differences observed for corn flour. One such factor might be the particle size distribution of the ground flour. This possibility is supported by the results of Naidu et al. (2007) in which particles passing through a 500 μm screen produced approximately 10% more ethanol than those passing through a 2000 μm screen. At limiting or 'optimal' enzyme concentrations, however, a difference caused by a greater proportion of fine particles might not be realized. In this experiment, glucoamylase was added well in excess (240 U $gram^{-1}$ corn flour dry weight) to ensure that substrate (i.e. particle surface area) was clearly the limiting factor.

Drought stressed kernels were more susceptible to α-amylase digestion (Figures 9A-9F). It is common to quantify starch hydrolysis in terms of reducing sugars produced relative to the total soluble carbohydrate content (% dextrose equivalents, % DE). Each glycosidic bond cleaved in the starch molecule generates an additional reducing group so an increase in reducing sugars (i.e. glucose) is indicative of α-amylase activity. When corrected for % dextrose equivalents at $t = 0$, drought stressed kernels exhibited a greater proportion of reducing sugars relative to total carbohydrates across all particle sizes and enzyme treatments. Unsieved flour from water stressed kernels exhibited 30% DE at 1 h compared to 17.4% DE for flour from irrigated kernels when treated with α-amylase alone (Figure 9A; P

= 0.031). Both treatments reached maximum values of 52% DE (water stressed) and 39% (irrigated) at 2.5 h ($P < 0.001$). When treated with protease for 2 h prior to the addition of α-amylase, the differences between water stressed and irrigated kernels were minimal at all sampling points (Figure 9B). Although still somewhat less, flour from irrigated kernels was hydrolyzed to a similar extent after 4 h compared to flour from water stressed kernels when pre-treated with protease ($P = 0.334$).

Particles passed over a 60 mesh screen were hydrolyzed at a slower rate relative to the unsieved samples, for both treatments (Figures 9C-9D). At 1 h, flour from water stressed kernels exhibited 17% DE while flour from irrigated kernels only reached 8% DE ($P = 0.013$). With the addition of protease, the irrigation treatments at 1 hr were not significantly different ($P = 0.188$). Hydrolysis of flour from irrigated kernels, however, was less than flour from water stressed kernels at 2.5 h and 4 h. The difference that remained between treatments after 2.5 h of hydrolysis could be a result of remaining proteins that were either not susceptible to the protease used in this study or were not accessible in the coarse particles.

Particles passing through a 60 mesh screen produced % DE values somewhat less than those obtained with unsieved particles (Figures 9E-9F). Hydrolysis values for irrigated and water stressed kernels were not significantly different after 1 hr, with or without pre-treatment with protease ($P = 0.231$). Beyond 1 hr, however, large differences in % DE values were observed.

In industrial scale dry-grind ethanol production, liquefaction by α-amylase is allowed to progress for at least 30 min at 90°C (Bothast and Schlicher, 2005). A period of 90 min at 90°C is commonly used in laboratory scale dry-grind ethanol experiments (Dien et al, 2002; Singh and Graeber, 2005). In the present study, the temperature was increased from 60°C to 90°C over a period of approximately 1 h after the addition of α-amylase. As such, treatment effects on the 2.5 h samples, which had been hydrolyzed for 90 min at 90°C, were analyzed in further detail. Particle size ($P < 0.0001$), enzyme treatment ($P = 0.0023$), and irrigation treatment ($P < 0.0001$) had significant effects on hydrolysis by α-amylase. Unsieved flour produced significantly greater % DE values than did the coarse and fine flours ($P < 0.05$). Averaged across all particle sizes and irrigation treatments, the addition of protease increased

% DE values by 8.1% relative to α-amylase hydrolysis alone ($P < 0.05$). Averaged across particles sizes and enzyme treatments, water stressed kernels resulted in 43% DE compared to 34% DE for irrigated kernels ($P < 0.05$). The interaction between enzyme treatment and irrigation treatment also was significant ($P = 0.004$). Averaged across all particle sizes, hydrolysis from water stressed kernels increased by less than 1% when treated with protease ($P = 0.400$). Hydrolysis from irrigated kernels increased by 22% when treated with protease prior to addition of α-amylase ($P = 0.066$). These results indicate that the arrangement or structure of protein bodies within the endosperm of irrigated kernels is remarkably different and is a major factor contributing to the limited hydrolysis of flour from irrigated kernels in comparison to that of water stressed kernels. Although % DE from irrigated kernels with the addition of protease was still less than that of water stressed kernels at 2.5 h, a similar extent of hydrolysis might be achieved with further optimization of protease dosage and treatment time.

The concentrations of reducing sugars and total carbohydrates (mg g^{-1} flour dry weight) at 2.5 h are presented in Table 4. Hydrolysis of coarse particles (>60 mesh) resulted in lower concentrations of reducing sugars and total carbohydrates relative to the other particle sizes. This is an important result in that irrigated kernels produced a greater proportion of particles passing over a 60 mesh screen. As such, the measured differences in starch hydrolysis might be a result of this larger ratio of coarse particles possessing reduced surface area. Fine particles (<60 mesh) produced the highest concentrations of total carbohydrates yet reducing sugar concentrations were similar or less than those of unsieved flour. These results are in general agreement with the results of Naidu et al. (2007) in which small particles supported high ethanol yields, presumably through an increased extent of starch hydrolysis. Ethanol plants typically mill the grain using hammer mills fitted with screens containing openings from 2 to 5 mm in width (Naidu et al., 2007). In our study, the largest opening was 1 mm, so starch hydrolysis and ethanol production on the industrial scale might be presented with a particle size distribution different that the one utilized in this study. Maisch (2003) reported a typical sieve analysis of dry-milled corn in which 64-86% of particles would be retained on a 60 mesh screen. Evidently, there is variability in particle

size distribution which might be attributable to genetics and the environment in which the grain is produced.

The use of α-amylase alone is not sufficient to provide ample fermentable sugars for yeasts in fuel ethanol production. Glucoamylase is added following α-amylase treatment to complete the saccharification process, typically occurring simultaneously with fermentation. In the present study, glucoamylase was added following 2.5 h of α-amylase treatment to determine the total extent of starch hydrolysis possible in each irrigation treatment. After 24 h of glucoamylase hydrolysis, concentrations of reducing sugars and total carbohydrates were higher than those after 2.5 h of α-amylase hydrolysis (Table 5). Averaged across irrigation and enzyme treatments, reducing sugar concentration increased by 34% relative to 2.5 h of α-amylase hydrolysis while total carbohydrate concentration increased by 20%. Reducing sugar concentrations were not significantly different between irrigation treatments when only α-amylase was used prior to glucoamylase ($P = 0.229$). Similarly, total carbohydrate concentrations were not significantly different ($P = 0.252$). Use of protease along with α-amylase increased reducing sugar concentration in both treatments. Protease pretreatment produced significantly higher reducing sugar concentrations from water stressed kernels relative to flour from irrigated kernels ($P = 0.024$). Total carbohydrate concentrations followed similar trends; use of protease increased total carbohydrate concentration in both treatments and more total carbohydrates released from water stressed kernel flour ($P = 0.091$).

Expressed as % DE released, irrigation treatment was not significant ($P = 0.374$) for α-amylase followed by glucoamylase hydrolysis. Also, there was no significant benefit from pre-treating flour from irrigated kernels with protease prior to addition of α-amylase ($P = 0.387$) when glucoamylase was used to complete the hydrolysis of starch. This is contrary to the results obtained while examining the effect of α-amylase in which flour from irrigated kernels gained the greatest benefit from protease pre-treatment. Use of protease and α-amylase followed by glucoamylase, however, resulted in significantly greater % DE released from water stressed kernel flour ($P = 0.018$). In general, the positive effect of protease pre-treatment measured in both the α-amylase experiments and the experiment using

glucoamylase to complete the hydrolysis of starch suggest that protein matrix seems to be a limiting factor to starch hydrolysis. Although not tested by this study, the effect of protease pre-treatment prior to addition of only glucoamylase should be examined to determine whether protein hydrolysis affects subsequent starch hydrolysis by α-amylase or glucoamylase in different ways.

Conclusions

Clearly, water stress during seed development has a dramatic effect on kernel dry matter accumulation. By decreasing the effective period of grain filling, potential sink capacity is not realized through limited synthesis of starch, protein, oil, and other seed constituents. While the results of this study suggest that kernel protein concentration is unchanged or increased by water stress, kernel density and hardness were decreased by the treatment. This disparity challenges conventional thinking about the role of protein concentration in maize seed composition and ethanol conversion efficiency. Further research is needed to evaluate the profile of endosperm storage proteins accumulated under water stress conditions, or how these proteins are arranged with starch granules in the endosperm matrix. For example, Woo et al. (2001) reported that the expression of mRNA encoding for the 22-kD α-zein is most abundant from 15 to 25 days after pollination. *Opaque2* (*o2*) maize mutants lacking in α-zein accumulation exhibit a low vitreousness phenotype similar to that observed in water stressed kernels. Therefore, water stress might act to limit α-zein accumulation in maize endosperm.

Water stress increased susceptibility to hydrolysis by α-amylase and glucoamylase as indicated by rate of hydrolysis. Additionally, when treated with protease, flour from water stressed kernels resulted in significantly higher total starch hydrolysis. Clearly, this result does not suggest that ethanol producers and other industries dependent on efficient starch hydrolysis should favor drought stressed maize grain as there are other negative consequences such as reduced yield and less starch per unit area. What the results do indicate, however, is that a maize hybrid developed for ethanol production might possess kernels of lower density that are more easily amenable to particle size reduction processes.

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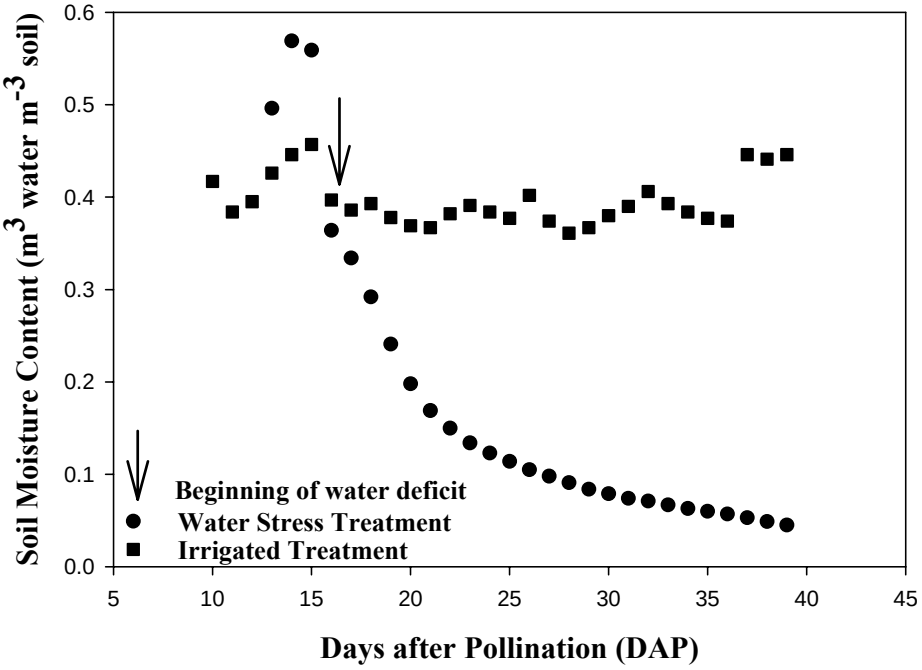


Figure 1. Volumetric soil moisture content (m^3 m^{-3} soil) for water stressed and irrigated treatments. Arrow denotes beginning of water stress treatment.

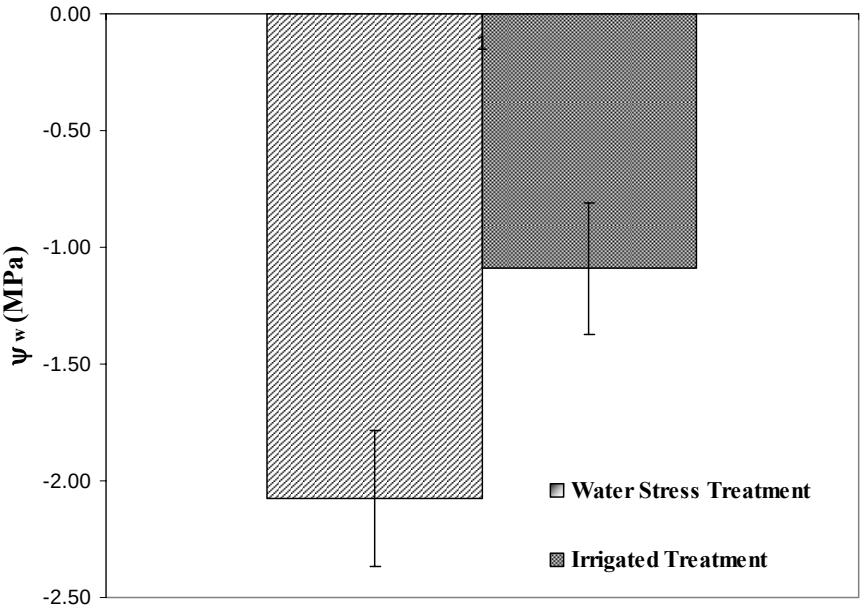


Figure 2. Mean leaf water potentials for water stress and irrigated treatments. Measurements were taken 23-25 days after pollination (7-9 days after beginning of water stress treatment). Means significantly different at ($P < 0.0001$). Error bars represent standard deviation of the mean.

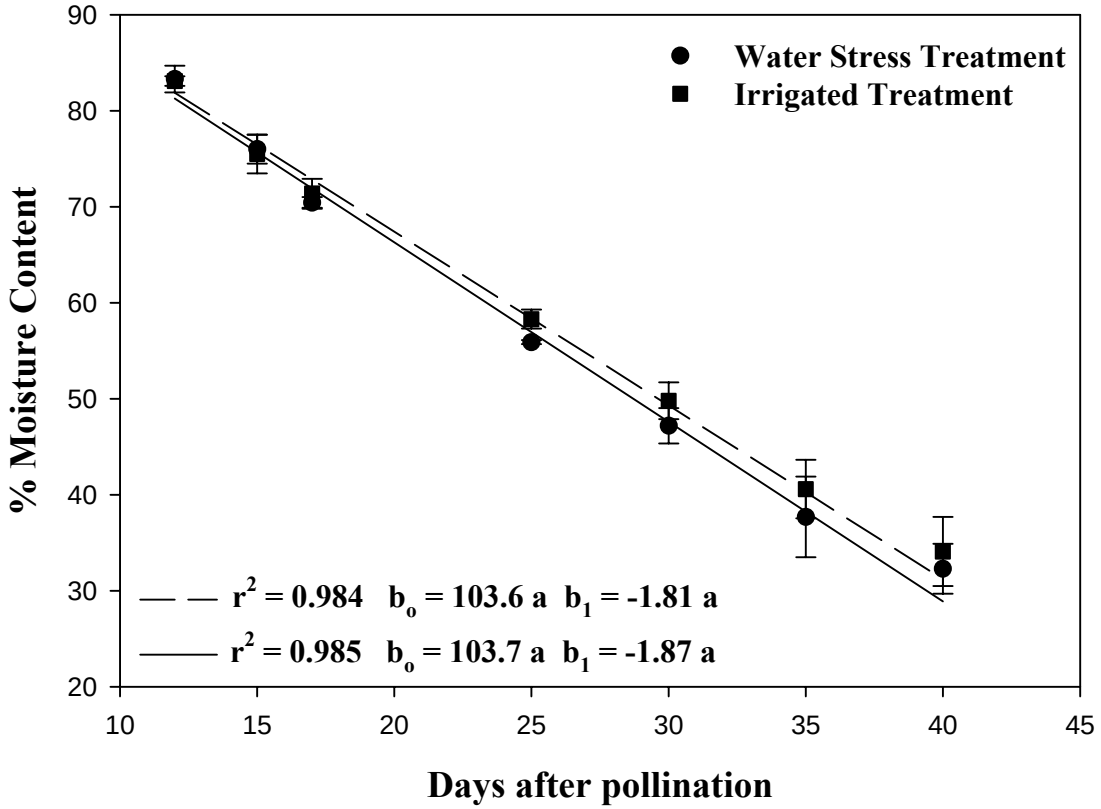
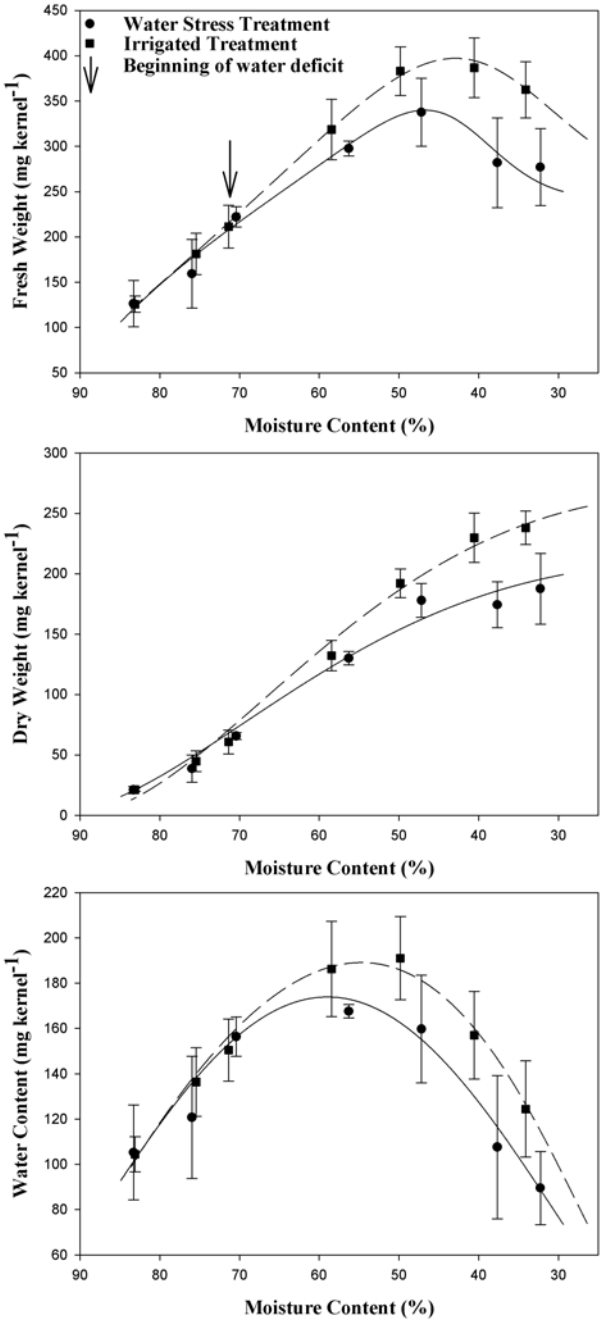


Figure 3. Percent moisture content (fresh-basis) of kernels versus days after pollination. Regression lines are of the form $y = b_o + b_1x$. Regression coefficients within each column followed by the same letter are not significantly different ($P < 0.05$). Error bars represent standard deviation of the mean.



Figures 4A-4C. Progression of kernel fresh weight, dry weight, and water content expressed on a % MC basis. Data points are the means of samples taken at 12, 15, 17, 25, 30, 35, and 40 days after pollination. Each point is the mean ± SD of 2-15 replications.

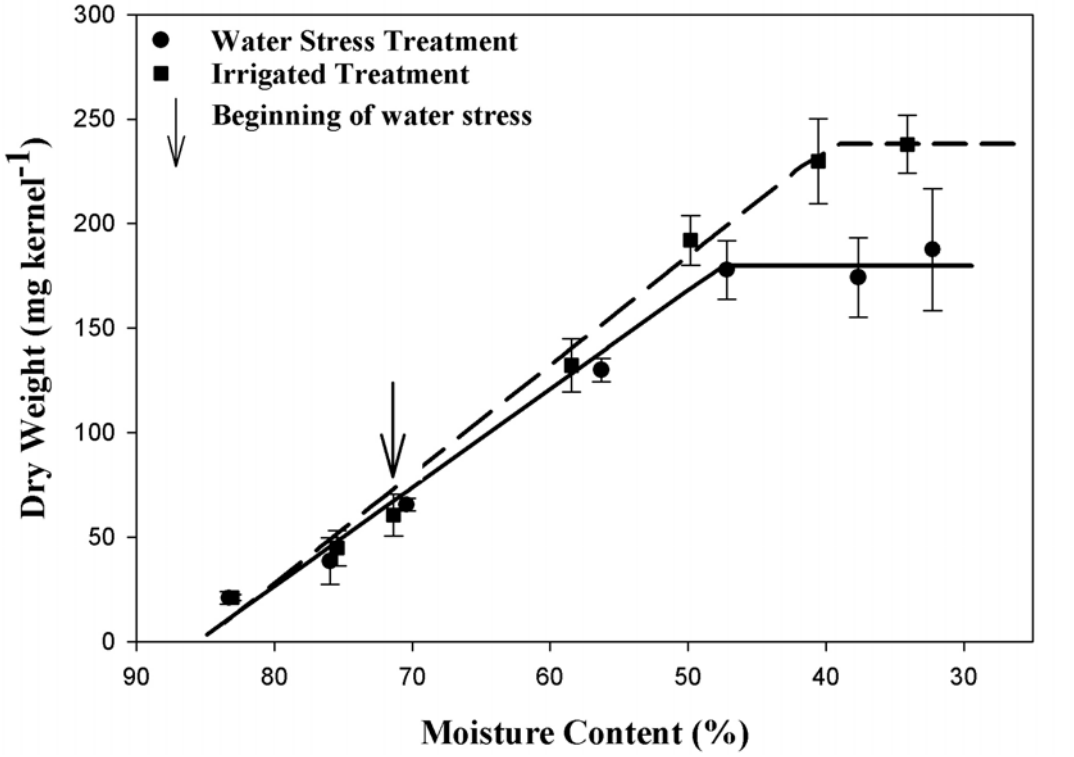


Figure 5. Bi-linear regression of dry weight accumulation in kernels of water stressed and irrigated plants. Data points are the dry weight means of samples taken at 12, 15, 17, 25, 30, 35, and 40 days after pollination. Error bars represent the standard deviation of the mean. Breakpoint of water stress treatment model was 47.5% MC (95% confidence limits: 41.5% MC - 53.6% MC). Breakpoint of irrigated treatment model was 39.7% (95% confidence limits: 37.5% MC - 41.9% MC).

Table 1. Kernel number per ear, dry weight, dry weight components, specific density, and starch granule diameter of kernels harvested at physiological maturity (~40 days after pollination).

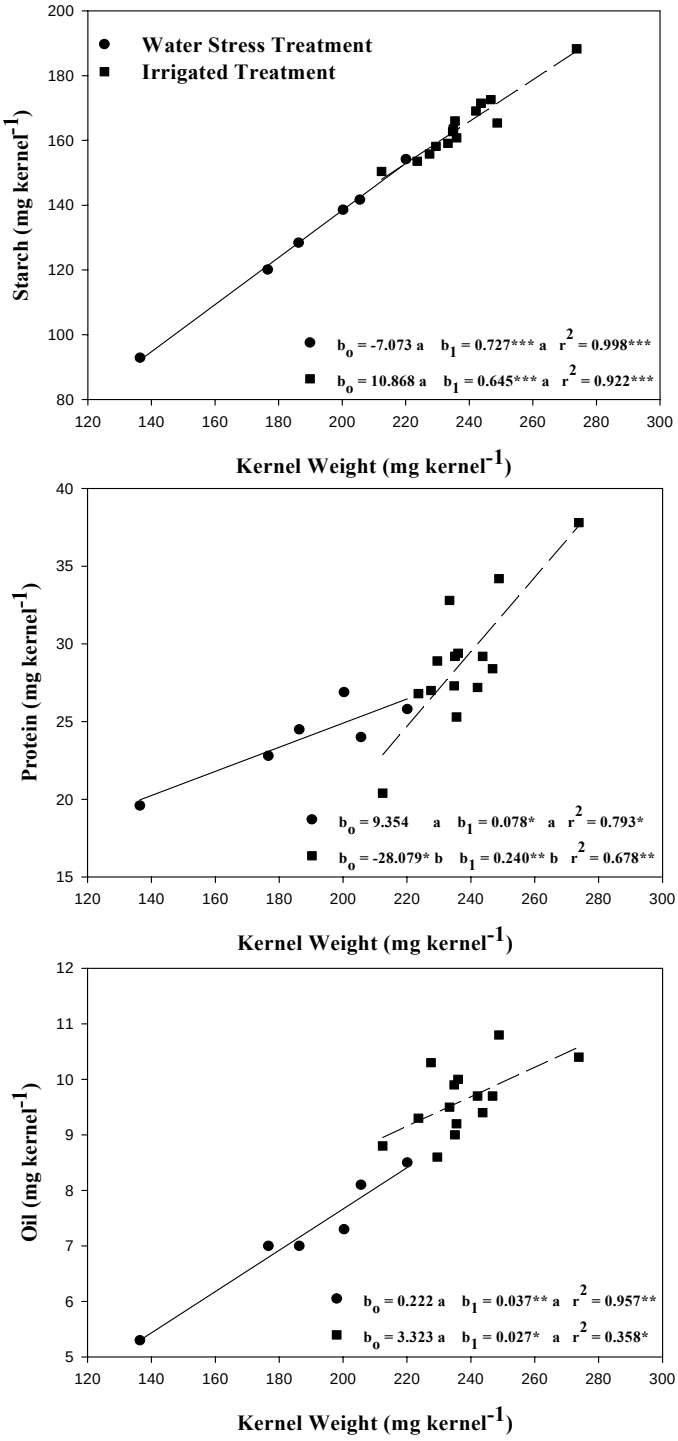
	Means		
Mature kernel characteristics	**Water Stress**	**Irrigated**	**P-value**
Kernel number	302	212	0.020
Kernel dry weight (mg kernel^{-1})	187.6	238.0	< 0.0001
Endosperm dry weight (mg kernel^{-1})	157.3	198.1	< 0.001
Embryo dry weight (mg kernel^{-1})	19.4	20.6	0.231
Pericarp dry weight (mg kernel^{-1})	10.0	13.7	< 0.0001
Specific density (g cm^{-3})	1.289[†]	1.314	0.047
Starch granule diameter (μm)	10.1	11.1	0.053

[†] Specific density means adjusted to 0% moisture content basis.

Table 2. Starch, protein, oil, apparent amylose, and soluble carbohydrate composition of mature B73 kernels grown under water stress and irrigated conditions. All means are expressed on a dry weight (0% MC) basis.

	Means[†]		
Kernel Quality Trait	**Water Stress**	**Irrigated**	**P-value**
% Starch (total)	68.9	69.1	0.599
% Starch (endosperm)	80.2	81.4	0.645
% Starch (embryo)	6.3	5.5	0.178
% Protein (total)	12.9	12.1	0.206
% Protein (endosperm)	11.5	10.7	0.295
% Protein (embryo)	20.1	20.0	0.857
% Oil (total)	3.9	4.1	0.064
% Apparent amylose	25.7	25.1	0.636
Reducing sugars (mg g^{-1})	6.1	9.4	< 0.0001
Total soluble carbohydrates (mg g^{-1})	33.5	37.3	0.023

[†] Values presented in this table are the means of at least three replications.



Figures 6A-6C. Starch, protein, and oil contents (mg kernel^{-1}) versus kernel weight (mg kernel^{-1}). Each data point represents a grain sample from a single plant. Regression lines are of the form $y = b_o + b_1x$. Regression coefficients within each column followed by the same letter are not significantly different ($P < 0.05$).

Table 3. Particle size distribution of ground corn flour from water stressed and irrigated kernels. Each value is the mean of three replications.

U.S. Sieve No.	Mesh opening (μm)	% Material retained on screens		P-value
		Water Stress	Irrigated	
18	1000	1.0	1.4	0.224
35	500	21.4	28.2	0.022
60	250	34.6	34.4	0.793
100	150	21.6	18.5	0.076
Pan	-	21.2	17.5	0.210

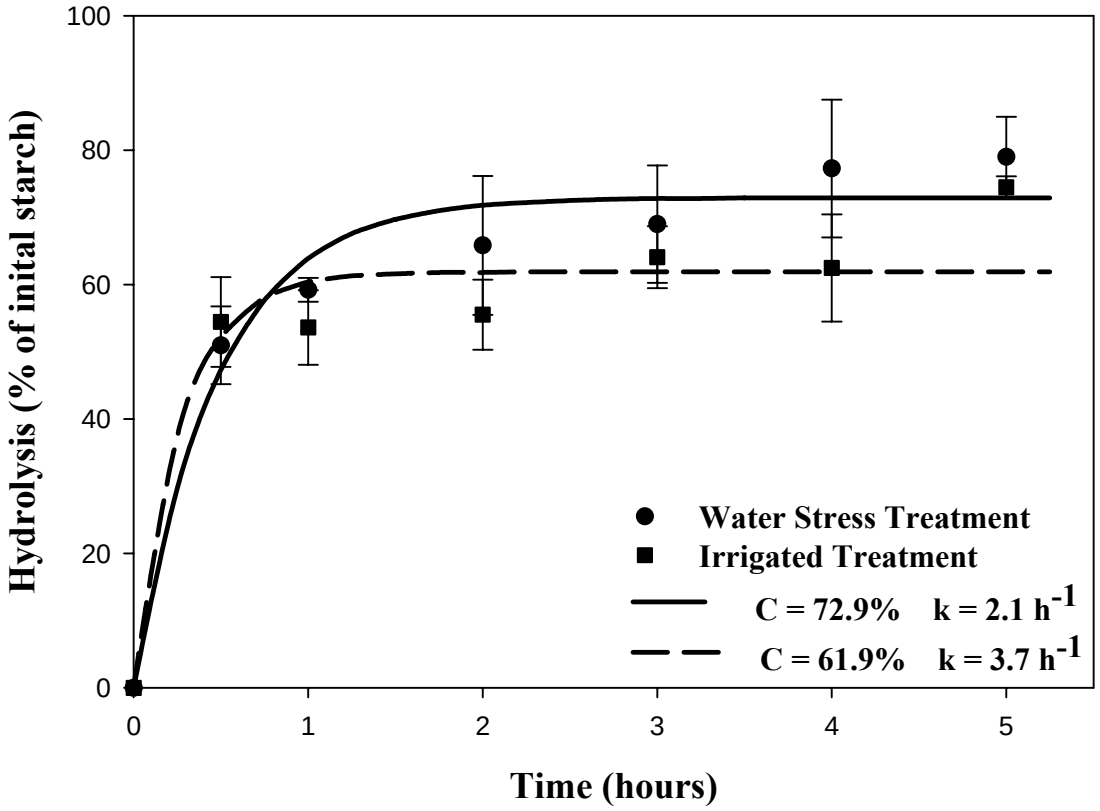


Figure 7. Glucoamylase susceptibility of corn flour from water stressed and irrigated kernels. Initial starch estimated from NIR spectroscopy measurements of whole kernels.

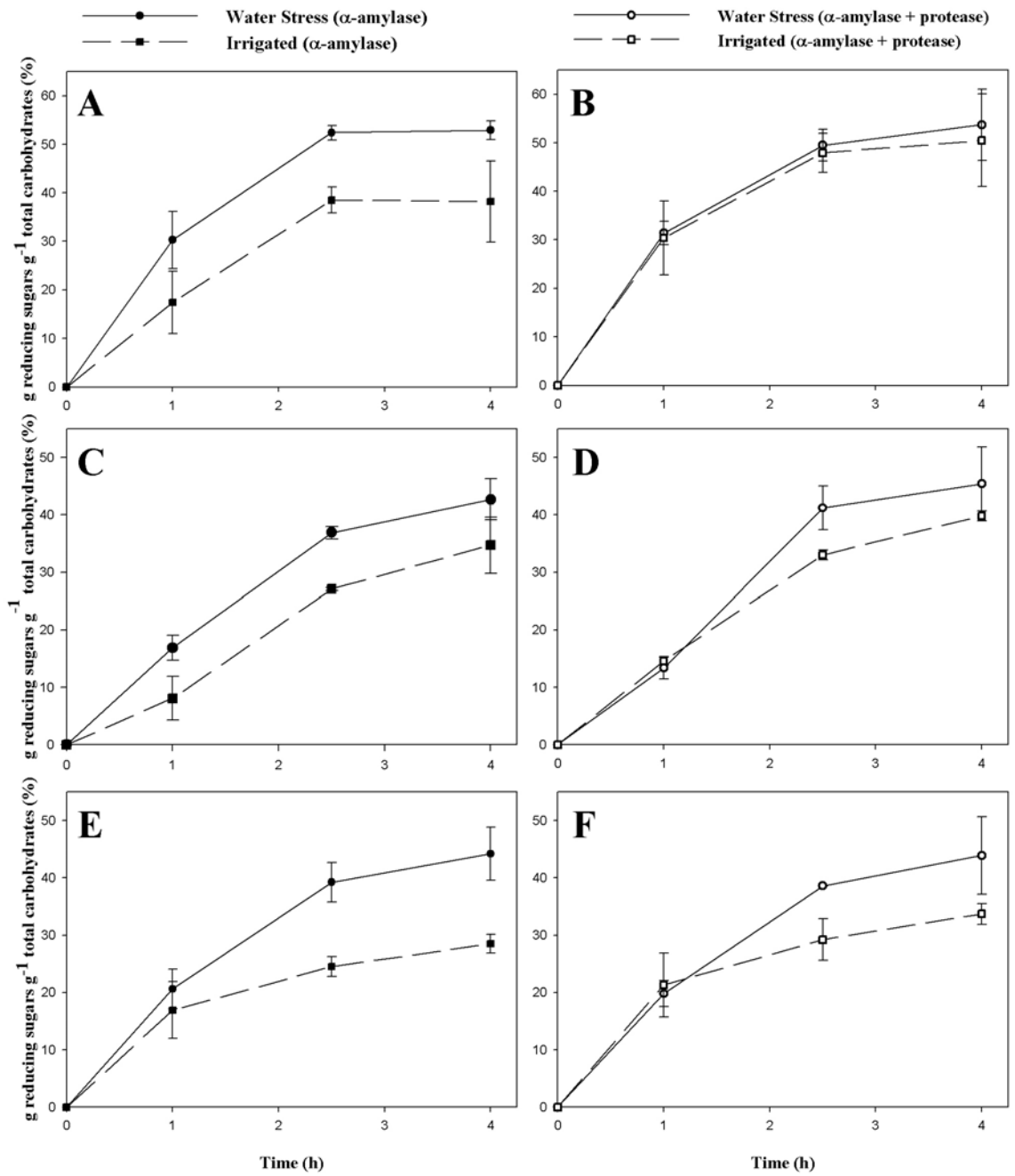


Figure 8A-8F. Hydrolysis of ground maize kernels from water stressed and irrigated kernels by bacterial α-amylase and protease. Whole ground kernels (Panels A & B), coarse particles (≥60 mesh; panels C & D), and fine particles (<60 mesh; panels E & F). Each point is the mean of three replications. Error bars signify standard deviation of the mean.

Table 4. Reducing sugar and total soluble carbohydrate concentration after 2.5 h of α-amylase hydrolysis.

Grain Fraction	Enzyme Treatment	Reducing Sugars		Total Carbohydrates	
		Water Stress	Irrigated	Water Stress	Irrigated
		— mg g^{-1} —		— mg g^{-1} —	
Unsieved	α-amylase	415.8 a[†]	384.3 a	589.4 bc	603.3 ad
	α-amylase + protease	407.2 a	437.7 a	599.3 acd	612.2 ac
Coarse (≥ 60 mesh)	α-amylase	287.9 b	288.6 bcd	530.0 bd	511.3 bcd
	α-amylase + protease	278.7 b	302.8 bcd	477.0 bd	511.5 bcd
Fine (< 60 mesh)	α-amylase	418.7 a	321.3 ad	783.0 ac	778.7 a
	α-amylase + protease	448.1 a	358.3 ac	823.3 a	781.8 a

[†] Means within a column followed by different letters are significantly different at $P < 0.05$.

Table 5. Total starch hydrolysis after 2.5 h of α-amylase hydrolysis followed by 24 h of glucoamylase hydrolysis.

Enzyme Treatment	Reducing Sugars		Total Carbohydrates		Dextrose Equivalents	
	Water Stress	Irrigated	Water Stress	Irrigated	Water Stress	Irrigated
	— mg g^{-1} —		— mg g^{-1} —		— % —	
α-amylase	484.7 a[†]	509.6 a	666.5 a	691.4 a	72.6 a	74.0 a
α-amylase + protease	655.2 b	547.1 a	787.3 b	729.7 a	83.2 b	74.9 a

[†] Means within a column followed by different letters are significantly different at $P < 0.05$.

Chapter 3. Maize Kernel Composition, Starch Digestibility, and Potential for Susceptibility to Aflatoxin Accumulation

A manuscript to be submitted to Phytopathology

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Abstract

Aflatoxin is a fungal secondary metabolite that contaminates agricultural commodities including maize (*Zea mays* L.). The plant physiological factors that facilitate mycotoxin accumulation in kernels are not well understood although the presence of aflatoxin is frequently associated with drought and high temperature during grain filling. Synthesis of aflatoxin *in vitro* has been linked to fungal α-amylase activity. If drought stress during grain filling increases susceptibility of starch granules to enzymatic hydrolysis, the increase in sugar availability could stimulate fungal growth and mycotoxin synthesis. The objectives of this study were to i) determine the impact of water stress on the potential for aflatoxin accumulation in maize kernels relative to irrigated kernels and ii) establish appropriate culture methods for measuring fungal α-amylase activity on isolated maize starch. Contrary to our expectations, the results of this study suggest that kernels of maize inbred B73 subjected to water stress during grain fill would be less susceptible to aflatoxin accumulation relative to kernels from irrigated plants. This result is not consistent with the general observation that mycotoxin accumulation increases under unfavorable environmental conditions. It is possible that kernels on well watered plants produce higher levels of endogenous α-amylase and soluble carbohydrates to support aflatoxin production. Future work should focus on quantifying α-amylase activity within infected kernels of water stressed and well watered plants.

Introduction

Aspergillus flavus Link ex. Fries frequently infects maize (*Zea mays* L.) kernels in regions that experience drought and high temperature during maize reproductive development. This fungus synthesizes aflatoxin, the most potent biologically derived carcinogen known. Human exposure to aflatoxin specifically promotes liver cancer, particularly in individuals infected with the hepatitis B virus (Wang et al., 1996). Also, aflatoxin has been linked to impaired growth in children (Gong et al., 2004). Mycotoxins such as aflatoxin promote morbidity and mortality in a number of animal species and are detrimental to livestock production by reducing weight gain. Due to the potential impact on human and animal health, the United States Food and Drug Administration (FDA) currently imposes a regulatory standard of 20 $\mu g\ kg^{-1}$ for aflatoxin levels in corn grain destined for interstate commerce. Many international grain export markets also have aflatoxin standards that are in some cases lower than the FDA requirements. For example, the European Union's total aflatoxin concentration limit of 4 $\mu g\ kg^{-1}$ (Wu, 2006). These standards limit the utility of grain produced in regions of the United States that experience recurrent drought and high temperatures, and consequently, aflatoxin contamination. As such, there are several government and university programs working toward characterizing and developing maize germplasm that is less susceptible or resistant to *A. flavus* infection and/or aflatoxin accumulation using traditional and molecular breeding approaches (Brown et al., 1999). Success in breeding for aflatoxin resistance will increase food safety and security, but a lack of understanding of the plant-pathogen-environment interactions that promote aflatoxin accumulation in maize impede germplasm improvement.

Despite nearly 50 years of research, the factors that regulate *A. flavus* - maize kernel interactions and aflatoxin production by the fungus are still not well understood. Aflatoxin accumulation in maize kernels has been associated with insect herbivory. It has been difficult, however, to establish a consistent relationship between insect feeding and aflatoxin accumulation (Windham et al., 1999; Wu, 2008). Pre-harvest aflatoxin contamination in maize is more consistently associated with drought and high temperature during reproductive development (Abbas et al., 2002; Munkvold, 2003). These conditions are common throughout the southern United States in which aflatoxin contamination is a frequent

detriment to crop quality in several economically important crop species. Severe droughts also occur sporadically throughout the Midwest Corn Belt and have resulted in widespread outbreaks of aflatoxin contamination as exemplified by the 1983 and 1988 droughts in Iowa. Less severe outbreaks occur regularly, and most recently in 2005.

Although drought and high temperature apparently enhance maize susceptibility to aflatoxin accumulation (Munkvold, 2003) and irrigation decreases susceptibility (Jones et al., 1981), the specific physiological mechanisms by which abiotic stresses enhance *A. flavus* infection and aflatoxin synthesis have not been resolved. Some of the mechanisms proposed that may either enhance or decrease susceptibility to aflatoxin accumulation include reactive oxygen species (reviewed in Chen et al., 2004a), a 14-kDa corn trypsin inhibitor with activity toward *A. flavus* α-amylase (Chen et al., 1999), and activity level of glyoxalase I (Chen et al., 2004b).

Drought stress during grain filling in maize has profound effects on kernel development. A water deficit during the early stages of kernel development is likely to result in kernel abortion and reduced kernel dry weight associated with fewer endosperm cells (Ober et al., 1991). The linear period of grain filling is also negatively affected by water stress. For example, when stressed at mid-grain fill, final kernel dry weight may be reduced by 36% relative to irrigated kernels (Westgate and Boyer, 1985). The metabolic changes that occur during kernel development under water or temperature stress conditions are not completely characterized. It is clear, however, that the decrease in final kernel dry weight results from an inhibition and/or premature termination of the primary storage reserves, starch and protein (Westgate, 1994). The accumulation of starch polymers (amylose and amylopectin) and proteins (primarily zeins) have temporally specific developmental patterns. So the timing and intensity of the imposed stress might have dramatically different effects on kernel metabolism. As such, perturbations to kernel metabolism and storage reserve accumulation are likely to affect final grain composition. The relative ratios of different compositional components within the kernel influence the physical and chemical properties of maize grain. One such property is that of susceptibility of kernel starch to enzymatic hydrolysis. Organisms such as *A. flavus* possess a wide array of hydrolases for accessing complex substrates such as starch. Therefore, increased or decreased susceptibility of starch

to enzymatic hydrolysis as a result of water stress during grain filling might influence the relative ability of *A. flavus* to produce aflatoxin in maize grain.

A review of previous literature supports the hypothesis that maize grain composition influences the potential for accumulation of aflatoxin and other mycotoxins such as fumonisin. When four endosperm types were compared for susceptibility to aflatoxin contamination, dent and flint types supported the greatest aflatoxin concentrations while high-amylose maize grain had an aflatoxin concentration only one-fifth that of the other two types (McMillian et al., 1981). Lillehoj et al. (1983) reported elevated aflatoxin concentrations in grain of a known susceptible hybrid (Mo17 x B73) and a waxy (~ 0% amylose) hybrid. Similarly, waxy hybrids supported 2-4 fold higher concentrations of fumonisin when compared to non-waxy hybrids with similar base genetics (Blandino and Reyneri, 2007). The significance of these results is that waxy starch granules are generally more susceptible to hydrolysis by α-amylase and glucoamylase while starch granules containing higher concentrations of amylose are more resistant to enzymatic degradation (Fuwa et al., 1977; Jane et al., 2003). Although the results suggesting a link between endosperm composition and aflatoxin accumulation might also be attributable to other susceptibility and resistance mechanisms, they indicate the need for further investigation into the role that substrate type and availability plays in supporting aflatoxin synthesis.

Other studies indicate that *A. flavus* α-amylase (*Amy1*) activity supports fungal growth and is essential for aflatoxin synthesis. α-amylase is an endoglucanase that randomly hydrolyses the α-1,4 glycosidic bonds in starch molecules. Woloshuk et al. (1997) demonstrated that expression of the *A. flavus ver1* gene, a structural gene in the aflatoxin biosynthetic pathway, was positively correlated to the activity of α-amylase in maize kernels and amylopectin culture filtrates. A strain of *A. flavus* with disrupted α-amylase activity was unable to produce aflatoxin in culture and only grew at 45% of the rate of the wild-type (Fakhoury and Woloshuk, 1999). Strains of *A. flavus* that are highly virulent in cotton (*Gossypium* spp.) generated 1.6 times greater α-amylase activity that less virulent strains (Brown et al., 2001). These results suggest that simple carbohydrates originating from hydrolysis of starch are important to fungal growth and necessary for aflatoxin synthesis. If indeed drought stress during grain filling results in starch granules with increased

susceptibility to α-amylase hydrolysis, the increase in sugar availability could stimulate *A. flavus* growth and aflatoxin synthesis.

Amylose content and granule size are two key factors implicated in granular starch digestibility. Generally, normal maize starches possess an amylose content of 20-30% of total starch on a dry weight basis. There are few reports of variation in granular starch digestibility among genotypes exhibiting similar amylose contents. Digestibility of solubilized starch, however, did not correlate well with amylose content in maize genotypes ranging from 23.4% to 34.6% amylose content (Uppalanchi, 2005). As such, a small change in amylose content in response to an environmental stress might not significantly alter digestibility properties. For example, when subjected to temperatures of 25^{o}C and 35^{o}C during grain filling, the average decrease of amylose content in response to temperature stress in two genotypes was only 2.3% (Lu et al., 1996).

Several studies have examined the effect of starch granule size on hydrolysis rate (Knutson et al., 1982; Franco et al., 1998; Kong et al., 2003). Clearly, smaller granules are hydrolyzed at an increased rate relative to larger granules. This property is likely due to the larger surface area to mass ratio of smaller granules which allows for greater enzyme adsorption (Kong et al., 2003). For example, as granule size decreases from 14.7 μm to 5.8 μm, the surface area increases from 2.7 cm^2 mg^{-1} to 6.9 cm^2 mg^{-1} (Knutson et al., 1982). This increase in surface area resulted in 45% more hydrolysis of the small starch granules relative to the large granules after four hours. Reports of environmental effects on starch granule size in maize are limited (Lu et al., 1996). High temperature generally decreases average granule size. Since drought stress during grain filling shortens dry matter accumulation, it is likely to limit starch granule size as well.

The results of previous experiments examining the role of α-amylase in fungal growth and toxigenesis were performed with either solubilized starch or autoclaved corn meal. These treatments destroy the granular, crystalline structure of starch that *A. flavus* would encounter in a maize kernel. As such, it might not be possible to extrapolate culture study results to natural processes *in planta*. Therefore, it is not clear whether *A. flavus* α-amylase displays differential activity toward granular starches, particularly starch granules from kernels grown under water stress conditions which might alter their size and structure.

The objectives of this study were to i) determine the potential for aflatoxin accumulation in water stressed and irrigated maize kernels and ii) evaluate culture methods for measuring fungal α-amylase activity toward isolated maize starch.

Materials and Methods

Experimental Design

Two treatments were included in the study; irrigated and water stressed. Plants were blocked by treatment due to the necessity to control water availability. Four replicates per treatment were sampled at physiological maturity. From these four replicates, three biological replicates (grain bearing ears) from each treatment were selected for use in subsequent experiments involving *A. flavus* growth and aflatoxin production.

Greenhouse Plant Care

Maize plants (inbred B73) were grown in the Iowa State University Department of Agronomy greenhouses in individual 19 L plastic pots containing a commercially available potting mix (Sunshine SB300 Universal, SunGro Horticultural, Bellevue, WA). Plant density was 2.4 plants m^{-2}. Growth conditions consisted of a 15-h photoperiod and $27^{o}C/18^{o}C$ maximum/minimum temperatures. Fertilizer (15-5-15; N-P-K) was injected into the irrigation water at a volumetric ratio of 1:40. After pollination, irrigation was managed automatically by GP1 data loggers and SM200 soil moisture sensors (Delta-T Devices, Cambridge, UK). Water was withheld from the water stress treatment beginning at 17 days after pollination (DAP) and continued until physiological maturity. Fertilizer was withheld from both treatments after 17 DAP to minimize effects due to differences in nutrient availability.

Kernel Weight

The plants were self- or sib-pollinated and ears were harvested at 40 DAP. Kernel number per ear was manually determined. Fifteen kernels from the middle one-third of the rachis were removed in a humidified box maintained at saturation vapor pressure and used for dry weight (DW), fresh weight (FW), and % moisture content (% MC) measurements.

Dry weights were obtained after drying the kernels to constant weight at 65°C. Percent moisture content (fresh basis) was calculated according to Equation 1.

$$\% \text{ MC} = \left(\frac{\text{FW} - \text{DW}}{\text{FW}} \right) \times 100 \quad (1)$$

Statistical Analyses

Kernel number, kernel dry weight, and % MC means for the two treatments were compared using two-sample t-tests with the assumption of equal variance (PROC TTEST; SAS 9.1, SAS Institute Inc., Cary, NC).

Grain Composition and Density

Total starch, oil, and protein concentrations were analyzed by near-infrared spectroscopy (NIR; Iowa State University Grain Quality Lab). Specific density (g cm^{-3}) was measured with an Accupyc 1330 nitrogen gas pycnometer fitted with a 35 cm^3 chamber (Micromeritics, Atlanta, GA). Total nitrogen content in endosperm and embryo tissue was determined by combustion analysis (Iowa State University Soil and Plant Analysis Laboratory). Total nitrogen was converted to protein using a conversion factor of 6.25.

Statistical Analyses

Grain composition and density means for the two treatments were compared using two-sample t-tests with the assumption of equal variance (PROC TTEST; SAS 9.1, SAS Institute Inc., Cary, NC).

Kernel Screening Assay and ELISA Detection of Aflatoxin

The kernel screening assay (KSA) method of Brown et al. (1993) was used to document the susceptibility of water stressed and irrigated kernels to aflatoxin accumulation. A 2 x 4 factorial experiment was designed in which irrigation treatment (water stressed vs. irrigated) and inoculation method were factors. The inoculation methods were embryo wounding, endosperm wounding, surface inoculation, and a control consisting of un-inoculated kernels. Three independent grain samples from each irrigation treatment were

surface sterilized with 700 ml L^{-1} ethanol for 1 min after which the kernels were allowed to air dry. Kernel moisture content was approximately 12% (fresh weight basis). Four kernels per treatment (irrigation treatment x inoculation method combination) replicate were inoculated with a spore suspension prepared from 12 day old cultures grown on potato dextrose agar (PDA) plates. The inoculum contained approximately 3.6 x 10^6 conidia ml^{-1} of *A. flavus* strain NRRL 3357 (USDA-ARS Culture Collection, Peoria, IL). Wounding inoculation treatments consisted of piercing the pericarp with a metal probe into either the underlying embryo or endosperm tissue followed by a brief dip into the spore suspension. Surface inoculated kernels were dipped directly into the inoculum without further treatment. Each replicate was placed into its own pre-weighed aluminum weighing dish. The dishes were placed into a dark chamber maintained at 30°C and saturation vapor pressure. The incubation period lasted for 7 days after which the kernels were removed and dried for 3 d at 65°C. Kernel dry weights were obtained to allow for calculation of aflatoxin concentrations in units of mass of toxin per mass of kernel dry weight. The experiment was replicated three times.

The dry kernels were homogenized for 2 min in 700 ml L^{-1} methanol followed by filtration of the supernatant through a 0.45 μm syringe filter. The filtrate was analyzed for aflatoxin concentration using a competitive enzyme-linked immunosorbent assay (ELISA) kit (MycoChek Aflatoxin; Strategic Diagnostics Inc., Newark, DE). The assay was performed according to the manufacturer's instructions and the sample absorbance results obtained at 650 nm were compared to a standard curve prepared from standards ranging from 0 to 80 ppb.

Statistical Analysis

The results were analyzed using PROC ANOVA (SAS 9.1; SAS Institute Inc., Cary, NC). Treatment means were compared using Tukey's studentized range method.

Semi-Quantitative Fungal Growth and Starch Hydrolytic Enzyme Assay

Starch agar plates were prepared according to Fakhoury and Woloshuk (1999) and Bluhm and Woloshuk (2008) with minor modifications. The medium consisted of 0.3 mol L^{-1} $(NH_4)_2SO_4$, 6 mmol L^{-1} Na_2HPO_4, 4 mmol L^{-1} $MgSO_4$, 7 mmol L^{-1} KCl, 66 μmol L^{-1} $FeSO_4$,

and 15 g L^{-1} agarose. The treatments consisted of commercially available starches (2 g L^{-1}) added to the medium in either solubilized or granular form. The starches used in the study were obtained from National Starch and Chemical Company (Bridgewater, NJ) and included ~ 0% amylose (Amioca), ~ 25% amylose (Melojel), ~ 50% amylose (Hylon V), and ~ 70% amylose (Hylon VII). All media preparations were autoclaved at 121°C for 1 h. The media containing solubilized starch had the starch added prior to sterilization. Media containing granular starch had the starch added after sterilization and after the sterilized solution had cooled to less than 60°C. After addition of granular starches to the culture medium, frequent stirring was employed to prevent settling of the starch granules. All media preparations were poured into standard 8.5 cm diameter Petri dishes to a depth of 0.5 cm. A 6 mm core of *A. flavus* strain NRRL 3357 was removed from 2 day old cultures grown on PDA and placed in the center of the starch agar plates. The plates were incubated at 30°C in the dark. Plates at 72, 96, and 120 h were flooded with iodine solution consisting of 0.3 mol L^{-1} KI and 5 g L^{-1} I_2. Unstained areas were indicative of starch hydrolytic activity (Figure 1). The extent of fungal colony growth was measured as the average diameter of the mycelial mat.

Results and Discussion

The kernels used in this study were at or past physiological maturity. Grain samples were harvested 40 days after pollination (DAP) at which point the % moisture content (% MC; fresh basis) of water stressed kernels was 34% while the % MC of irrigated kernels was 38% ($P = 0.03$). Although the moisture content of irrigated kernels was significantly higher, black-layer was apparent in both treatments at harvest indicating that physiological maturity (maximum kernel dry weight) had been reached by both irrigated and water stressed kernels. Additionally, the results of a related experiment indicate that grain fill in irrigated kernels of inbred B73 terminates at approximately 40% MC under the greenhouse conditions employed in this study.

Kernels from water stressed plants accumulated only 174 mg of dry matter per seed in comparison to 233 mg per seed in kernels from irrigated plants (Table 1; $P = 0.019$). This reduction in dry matter accumulation was a result of a shortened duration of grain filling rather than a difference in rate of accumulation (data not shown). Kernel number per ear was

not significantly different between treatments; kernel number averaged across treatments was 291 ($P = 0.469$). Kernel density means were not significant ($P = 0.227$). The reduction in kernel weight in the water stress treatment resulted in lesser total amounts of starch, protein, and oil accumulated within the kernel.

The water stress treatment changed kernel composition (Table 2). Total protein concentration increased to 13.6%, compared to 12.2% in irrigated kernels ($P = 0.019$). Total starch concentration in the kernel was not significantly different between treatments ($P = 0.469$). Within the endosperm protein concentration was increased by water stress from 11.3% to 12.9% ($P = 0.022$). Oil concentration was similar in water stressed kernels (3.8%) and irrigated kernels (4.1%; $P = 0.070$). These results do not necessarily point to an increased potential for fungal α-amylase activity and aflatoxin accumulation; however, a related study indicated that solubilized starch from water stressed kernels was much more susceptible to α-amylase hydrolysis (increased by 28% after 2.5 h; Chapter 2). Whether results on solubilized starch from ground kernels translate to granular starch hydrolysis by fungal enzymes has yet to be determined.

Surprisingly, inoculated kernels from water stressed plants accumulated less aflatoxin after 7 days of fungal infection than did kernels of well watered plants (Tables 3 and 4; $P < 0.001$). Averaged across all inoculation methods, water stressed kernels had an aflatoxin concentration of 102 $\mu g\ g^{-1}$ while irrigated kernels accumulated 233 $\mu g\ g^{-1}$. Although aflatoxin accumulation varied considerably by experimental replication ($P = 0.003$), kernels from irrigated plants were consistently more susceptible to aflatoxin accumulation.

Inoculation method also was a significant factor in the analysis of variance. The method used is a particularly valuable approach to assess where resistance mechanisms might be localized within the kernel. Embryo wounding supported the highest concentrations of aflatoxin (Table 5). Averaged across all replications embryo wounding resulted in 274 $\mu g\ g^{-1}$ aflatoxin. This level was similar to that of endosperm wounding but significantly higher than the surface and un-inoculated treatments ($P < 0.01$). Endosperm wounding also supported high concentrations of aflatoxin (215 $\mu g\ g^{-1}$) but was not significantly different from all other inoculation methods ($P < 0.01$). At $P < 0.1$, endosperm wounding was not significantly different from embryo wounding but was significantly

different from surface wounding and the control kernels. Fungal growth was visible and aflatoxin was detected in the un-inoculated kernels as well. This background level of infection is likely a result of handling these kernels in the incubation chamber along with the other treatments. Independent culture tests of un-inoculated kernels indicated that the kernels used in this study did not contain backgrounds levels of *A. flavus* (data not shown). Aflatoxin levels in the surface inoculated kernels were the lowest of all treatments (91 $\mu g\ g^{-1}$) and not significantly different from the non-inoculated kernels. These results confirm that an intact pericarp is an important component of overall resistance to fungal infection and consequent aflatoxin accumulation. There was no interaction between irrigation treatment and inoculation method ($P = 0.569$) which suggests that water stress per se did not render aflatoxin accumulation more responsive to the chosen method of inoculation.

Although the aflatoxin levels recorded in this study are in some cases nearly 14,000-fold higher than the FDA action level of 20 $\mu g\ kg^{-1}$, our results are in general agreement with those reported for the kernel screening assay by Brown et al. (1993). In their study, aflatoxin concentrations ranged from 23 $\mu g\ g^{-1}$ to over 44,000 $\mu g\ g^{-1}$ depending on genotype and kernel treatment. Clearly, this approach indicates potential for aflatoxin accumulation using a concentrated inoculum load and ideal conditions for fungal growth and aflatoxin accumulation. Although likely to be less than what is measured in the kernel screening assay, grain aflatoxin concentration under field conditions can vary widely depending on genotype, year, and location. For example, Campbell and White (1995) reported mean aflatoxin concentrations that ranged from 99 ng g^{-1} to over 37,000 ng g^{-1}.

A. flavus was grown on agar plates containing starches of different amylose contents to establish whether there are differences in fungal growth and hydrolysis of solubilized and granular starches over a full range of amylose contents. Having this information from culture studies is important for designing further experiments using intact maize kernels from diverse germplasm sources. Generally, fungal growth as measured by mycelial mat diameter was not significantly different among starch amylose contents when averaged across starch form (Table 6; $P < 0.05$). There was no consistent ranking of starch amylose content for fungal growth at all time points. As an exception to the lack of significance measured at 72 h and 96 h, the mean diameter of mycelia on plates containing 50% amylose starch was

significantly less than the other starch amylose contents at 120 h ($P < 0.05$). Starch form (solubilized versus granular) had a significant impact on fungal growth at all time points ($P < 0.05$). Generally, solubilized starch supported faster mycelial growth (mat diameter) than did granular starch. Although granular starches supported larger mycelial diameters in some cases, the mycelial growth was visually less dense and resulted in fewer visible sclerotia (Figure 2). Thus, radial growth measurements alone provide only limited insight relating starch type and fungal biomass growth. Other methods such as quantitative real-time PCR (qPCR) are likely to be more accurate for quantifying total fungal biomass (Niessen, 2007). For example, Mayer et al. (2003) reported the detection and quantification of *A. flavus* using the *nor-1* gene as a marker of fungal biomass. Copy number of the *nor-1* gene correlated with fungal colony forming units (CFU); however, gene copy number was always higher due likely to the multinucleate nature of fungal cells.

Differences in susceptibility to hydrolysis by fungal amylase were more evident using the starch plate approach (Figure 1). The diameters of unstained areas in the center of starch plates indicated that the solubilized waxy (amylose-free) starch supported significantly more hydrolysis than the high amylose starches (Table 6 and Figure 1). The average diameter of starch hydrolysis in the waxy starch plates at 96 h was 4.8 cm. This diameter was two times greater than 25% amylose starch and approximately two and a half times greater than the starch hydrolysis of 50% and 70% amylose starches ($P < 0.05$). By 120 h, differences in the extent of starch hydrolysis were less consistent. High amylose starch (~ 70%) exhibited extensive starch hydrolysis and was similar to waxy starch at 120 h. There was no evidence of starch hydrolysis in plates containing granular starch (as visualized by iodine staining). It is likely that the intact granules were incompletely hydrolyzed by fungal α-amylase, so that the starch molecules were still able to bind iodine. Because this technique only measured complete starch hydrolysis, it may not be suitable for quantifying potential for fungal growth and α-amylase in infected kernels containing intact starch granules.

Conclusions

Aflatoxin is an important food safety issue in many maize growing regions of the world. No commercially acceptable germplasm sources containing adequate levels of resistance to aflatoxin accumulation are available. The influence of environment on infection by *A. flavus* and aflatoxin accumulation is an impeding factor in characterizing and developing germplasm for resistance. Increased attention should be given to understanding the specific mechanisms by which drought stress increases potential for aflatoxin accumulation. Since a growing body of evidence suggests that fungal α-amylase activity is a prerequisite for aflatoxin production, kernel composition and starch digestibility as perturbed by reduced water availability might be important factors influencing the relative ability of *A. flavus* to grow and produce aflatoxin in maize kernels. The results of this study suggest that water stressed B73 kernels are less susceptible to aflatoxin accumulation than irrigated kernels. Future studies should quantify α-amylase activity in these kernels to determine if this hypothesis is supported by the aflatoxin concentration results.

The association between drought and elevated concentrations of aflatoxin in maize kernels under field conditions is extremely consistent. Few studies, however, have documented the effect of drought on aflatoxin contamination under controlled conditions. Payne et al. (1986) grew maize plants under managed field conditions and reported that aflatoxin concentration in grain from irrigated plants was only significantly less than the aflatoxin concentration of drought-stressed grain when silk inoculation was used. To our knowledge, there are no reports documenting a difference in post-harvest aflatoxin accumulation in kernels harvested from water stressed and irrigated plants. The results of this study should not be extrapolated to field conditions. The results may be more relevant to post-harvest storage of grain and the employed methodology more appropriate for maize germplasm with kernel composition optimized for uses such as conversion to ethanol (high starch digestibility). It is also important to note the observed differences between treatments in this study might be a genotypic characteristic unique to B73 that warrants further investigation along with other germplasm.

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Table 1. Kernel number per ear, dry weight, and specific density of kernels harvested at physiological maturity (~40 days after pollination) and used for aflatoxin susceptibility studies.

	Means		
Mature kernel characteristics	**Water Stress**	**Irrigated**	**p-value**
Kernel number	293	288	0.469
Kernel dry weight (mg kernel^{-1})	174.3	233.4	0.019
Specific density (g cm^{-3}) †	1.318	1.327	0.227

† Specific density means adjusted to 0% moisture content basis.

Table 2. Starch, protein, and oil concentrations of mature B73 kernels grown under water stress and irrigated conditions. All means are expressed on a dry-weight (0% MC) basis.

Kernel Quality Trait	Means[†]		p-value
	Water Stress	Irrigated	
% Starch (total)	68.7	68.8	0.469
% Protein (total)	13.6	12.2	0.019
% Protein (endosperm)	12.9	11.3	0.022
% Protein (embryo)	20.3	20.6	0.415
% Oil (total)	3.8	4.1	0.070

[†] Values presented in this table are the means of at least three replications.

Table 3. Analysis of variance for kernel screening assay comparing potential for aflatoxin accumulation in irrigated and water stressed maize kernels.

	ANOVA		
Source of Variation	**df**	**ms**	**Pr > F**
Irrigation treatment	1	305814	0.0003
Inoculation method	3	149078	0.0003
Replication	2	132607	0.003
Irrigation treatment x Inoculation method	3	13878	0.569
Irrigation treatment x Replication	2	9696	0.625
Inoculation method x Replication	6	21550	0.402

Table 4. Aflatoxin concentration means (μg g^{-1}) for irrigated and water stressed B73 kernels. Each treatment replication consisted of 16 kernels.

	Aflatoxin Concentration (μg g^{-1})			
Treatment	**Rep 1**	**Rep 2**	**Rep 3**	**Means**[†]
Irrigated	319	213	167	233 a
Water Stress	188	43	78	102 b

[†] Means within a column followed by different letters are significantly different at $P < 0.001$.

Table 5. Aflatoxin concentration means (μg g^{-1}) by kernel inoculation method. Each inoculation method replication consisted of 8 kernels.

	Aflatoxin Concentration (μg g^{-1})			
Inoculation Method	**Rep 1**	**Rep 2**	**Rep 3**	**Means**[†]
Embryo Wounding	321	267	233	274 a
Endosperm Wounding	377	134	133	215 ab
Surface	131	27	116	91 b
Un-inoculated	187	84	9	93 b

[†] Means within a column followed by different letters are significantly different at $P < 0.01$.

Table 6. Growth of *A. flavus* and starch hydrolysis on starch agar plates varying in amylose content. Each value is the mean of three replications ± standard deviation. Starch hydrolysis was not determined in plates containing granular starch (n.d.).

		72 h		96 h		120 h	
Amylose content	**Starch form**	**Growth**	**Hydrolysis**	**Growth**	**Hydrolysis**	**Growth**	**Hydrolysis**
		— cm —		**— cm —**		**— cm —**	
0%	Soluble	4.4 ± 0.3	1.9 ± 0.3	4.9 ± 0.5	4.8 ± 0.4	5.7 ± 0.4	5.1 ± 0.5
25%	Soluble	4.7 ± 0.2	1.3 ± 0.1	5.6 ± 0.3	2.4 ± 0.1	6.2 ± 0.2	2.8 ± 0.3
50%	Soluble	4.8 ± 0.2	1.1 ± 0.03	4.8 ± 0.3	1.9 ± 0.4	5.4 ± 0.0	4.3 ± 0.7
70%	Soluble	5.0 ± 0.4	1.1 ± 0.2	5.9 ± 0.3	1.5 ± 0.1	6.2 ± 0.5	6.0 ± 0.3
0%	Granular	4.6 ± 0.1	n.d.	5.9 ± 0.5	n.d.	8.0 ± 0.0	n.d.
25%	Granular	4.0 ± 0.2	n.d.	5.3 ± 0.3	n.d.	8.0 ± 0.0	n.d.
50%	Granular	4.3 ± 0.4	n.d.	6.2 ± 0.6	n.d.	6.9 ± 0.4	n.d.
70%	Granular	3.9 ± 0.2	n.d.	5.4 ± 0.2	n.d.	7.1 ± 0.2	n.d.

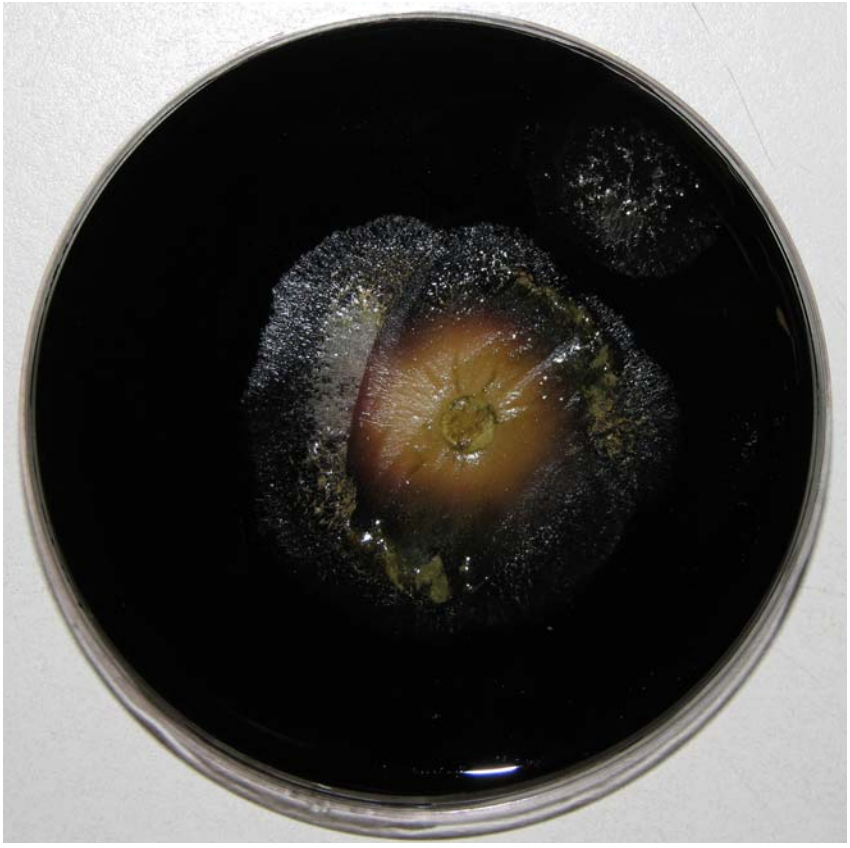

Figure 1. Starch plate used for assessing relative ability of *A. flavus* to grow on and hydrolyze starches of different amylose contents. Plate shown contains solubilized waxy (~ 0% amylose) corn starch stained after 72 h of growth. Note the unstained area indicative of starch hydrolysis in the center of the plate.

Figure 2. Growth of *A. flavus* on solubilized starch plate (left) versus granular starch plate (right). Plates contain waxy (~ 0% amylose) starch. Photo taken after 120 h of growth.

Chapter 4. Developmental Patterns of Gene Expression in Water Stressed and Irrigated Maize Kernels

A manuscript to be submitted to Crop Science

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Abstract

Gene expression profiling is a particularly valuable approach to identify transcriptional processes that regulate metabolic and developmental responses to high temperature and water deficit stress in maize (*Zea mays* L.) kernels. Because such abiotic stresses can alter kernel development dramatically, the relevance of gene expression data may be difficult to assess if profiles are not examined with respect to key physiological processes in the developing seed, such as kernel water relations. Our hypotheses were that i) a shortened duration of grain filling reflects the decreased expression of key genes involved in starch and storage protein biosynthesis, and ii) the shifts in kernel water content in water stressed kernels induce decreased transcription of those key genes.

We quantified the expression of three genes related to starch biosynthesis and storage protein synthesis in developing endosperms from irrigated and water stressed plants of inbred B73. The water deficit, imposed by withholding water prior to rapid grain filling, shortened the duration of dry matter accumulation resulting in a 21% reduction in final seed dry weight. Starch, protein, and oil concentrations, however, were not significantly affected. The results of a related study indicate that susceptibility of kernel starch to enzymatic hydrolysis by glucoamylase was enhanced by 17.8% in the stressed kernels relative to the controls, which suggests more subtle changes in grain physical properties or composition of starch granules might have occurred. This manuscript reports a preliminary analysis of gene expression compared on the bases of kernel moisture content and % of maximum kernel dry weight. *Shrunken2*, a gene in the maize starch biosynthetic pathway followed similar patterns of expression in both irrigated and water stressed treatments. Expression declined following

maximum kernel water content and was minimal only after physiological maturity had been reached. Expression of *az19B1* and *Opaque2*, two genes related to storage protein synthesis, followed similar patterns. A late peak in expression was observed in the irrigated treatment while expression of these storage protein synthesis genes in the water stressed treatment declined.

Introduction

Gene expression analysis has emerged as a powerful tool of modern molecular biology. This methodology is a particularly valuable approach to quantifying genome-wide transcriptional differences between environmental stresses, stage of development, and genotype. For example, cDNA microarray technology has been used to analyze the influence of environmental stresses on early maize reproductive development (Zinselmeier et al., 2002; Andjelkovic and Thompson, 2006; Zhuang et al., 2007). Although specific transcriptional responses have been documented in water stressed kernels (Yu and Setter, 2003; Andjelkovic and Thompson, 2006; Zhuang et al., 2007) it has been difficult to use such results to direct crop physiology research and breeding activities. Aligning gene expression data to a common developmental scale is of particular importance, especially if comparisons between environmental treatments or genotypes are required. For example, seed development can be expressed relative to days after pollination, thermal time (i.e. growing degree days), or seed water content. Borrás and Westgate (2006) showed that seed water content expressed as percent moisture content (% MC) was an accurate indicator of seed development in maize. When they compared dry matter accumulation on a scale of declining seed moisture content, common developmental patterns emerged among genotypes and treatments. Despite dramatic variation in the rate and duration of grain filling, all genotypes initiated rapid dry matter accumulation at about 80% MC and ceased accumulation at approximately 35% MC under well watered conditions. Also, final kernel weight was linearly related to maximum kernel water content achieved during grain filling (Borrás and Westgate, 2006). Their results implied that seed dry matter accumulation and water relations may share common regulatory mechanisms which are not obvious when kernel development is expressed as a function of days after anthesis.

Studies of seed gene expression under environmental stress typically seek to identify genes that might be targeted for improved crop responses to environmental perturbations (Cushman and Bohnert, 2000; Barnabás et al., 2008). Such efforts may lead to erroneous conclusions, however, if shifts in gene expression reflect an accelerated developmental response rather than one specific to environmental queues. Therefore, it is essential to compare gene expression in a way that allows parsing of developmentally and environmentally specific patterns of gene expression. We hypothesize that aligning stress treatments using declining grain % MC as a common scale will allow for the comparison of gene expression patterns; however, it is not known if gene expression follows kernel water content. The objectives of this study therefore were i) to assess differences in gene expression profiles for key genes involved in starch and storage protein biosynthesis in relation to kernel water content expressed in % MC and ii) to determine how perturbations in kernel composition or physical properties measured in Chapter 2 are related to changes at the transcriptional level for related genes. Based on the results of Chapter 2, fourteen genes involved in storage product (starch, protein, and oil) synthesis and seed maturation were selected for targeted expression analysis using quantitative RT-PCR. Relative expression levels of these genes were compared for kernels which developed under irrigated and water stressed conditions.

Materials and Methods

Experimental Design

Two treatments were included in the study; irrigated and water stressed. Plants were blocked by treatment due to the necessity to control water availability. Within each block, plants were designated for specific sampling dates which were randomized to minimize positional effects. Three replicates per treatment were designated for each sampling date prior to physiological maturity. Five replicates per treatment were sampled at physiological maturity. The experiment was repeated two times and data for common sampling dates were pooled.

Greenhouse Plant Care

Maize plants (inbred B73) were grown in the Iowa State University Department of Agronomy greenhouses in individual 19 L plastic pots containing a commercially available potting mix (Sunshine SB300 Universal, SunGro Horticultural, Bellevue, WA). Plant density was 2.4 plants m^{-2}. Growth conditions consisted of a 15-h photoperiod and 27°C/18°C maximum/minimum temperatures. Fertilizer (15-5-15; N-P-K) was injected into the irrigation water at a volumetric ratio of 1:40. After pollination, irrigation was managed automatically by GP1 data loggers and SM200 soil moisture sensors (Delta-T Devices, Cambridge, UK) indicated the range of soil moisture content values for irrigated and water stress treatments. Water was withheld from the water stress treatment beginning at 17 days after pollination (DAP) and continued until physiological maturity. Fertilizer was withheld from both treatments after 17 DAP to minimize effects due to differences in nutrient availability.

Kernel Development and Sampling

The plants were self- or sib-pollinated and ears were harvested at six intervals from 12 to 35 DAP. Kernel samples for gene expression profiling (a minimum of two replicates per treatment and sampling date) were excised from the ear and separated into embryo and endosperm fractions. These tissues were immediately frozen in liquid N_2. Samples were stored at -80°C until further analysis. Ears from each treatment harvested at each sampling date were placed in air-tight plastic bags for transport to the laboratory. Fifteen kernels from the middle one-third of the rachis were removed in a humidified box maintained at saturation vapor pressure and used for dry weight (DW), fresh weight (FW), water content (WC), and % moisture content (% MC) measurements. Dry weights were obtained after drying the kernels to constant weight at 65°C. Percent moisture content (fresh basis) was calculated according to Equation 1.

$$\% \text{ MC} = \left(\frac{\text{FW} - \text{DW}}{\text{FW}} \right) \times 100 \qquad (1)$$

RNA extraction and quantification

The extraction method used was based on protocols developed by Prescott and Martin (1987) and further refined at the University of Arizona (Leiva et al., 2002). Approximately 100 mg of frozen endosperm or embryo tissue was quickly ground in liquid N_2 using a mortar and pestle. The tissue was transferred to a microcentrifuge tube followed by the addition of 200 µl of extraction buffer. The extraction buffer consisted of 50 mmol L^{-1} tris(hydroxymethyl)aminomethane (pH 8.0), 150 mmol L^{-1} LiCl, 5 mmol L^{-1} EDTA (pH 8.0), and 10 g L^{-1} sodium dodecyl sulfate. All stock solutions were made on diethyl pyrocarbonate (DEPC) treated water. Phenol:chloroform:isoamyl alcohol (200 µl, pH 7.9) was added following the extraction buffer. This mixture was shaken and allowed to incubate on ice for 5 min. The mixture was transferred to a Phase Lock Gel tube (heavy formulation; 5 PRIME, Gaithersburg, MD) and centrifuged for 20 min at 2500 x *g* and 4°C. An additional 200 µl of phenol:chloroform:isoamyl alcohol was added and the sample was centrifuged again. The samples were again incubated on ice for 5 min after the addition of 200 µl chloroform. The samples were centrifuged as previously described to separate the organic and aqueous phases.

The aqueous phase from each Phase Lock Gel tube was poured into a second tube. Trizol Reagent (1 ml; Invitrogen, Carlsbad, CA) was added and used according to the manufacturer's instructions. The aqueous phase following Trizol extraction was transferred to a new tube. The monovalent cation concentration was adjusted with the addition of 5 mol L^{-1} ammonium acetate to a final concentration of 2.5 mol L^{-1}. Glycoblue co-precipitant (Ambion, Austin, TX) was added as a visual marker of RNA precipitation. Isopropyl alcohol (0.5 ml) was added followed by incubation at -20°C for 15 min. Samples were centrifuged for 30 at 2500 x *g* and 4°C. The resulting RNA pellet was washed with 1 ml ethanol followed by brief air drying. RNA was resuspended in 50 µl of 1 $mmol^{-1}$ sodium citrate (pH 6.4) and stored at -80°C.

Contaminating genomic DNA was removed from RNA using DNase I treatment. Initial nucleic acid concentration of the resuspended samples was estimated using ultraviolet (UV) spectrophotometry at 260 nm. Sample concentration was adjusted to 0.2 µg $µl^{-1}$ using

DEPC treated water. DNase I buffer (5 μl) and 1 μl (2 U) Recombinant DNase I (Ambion, Austin, TX) were added to 50 μl of sample followed by incubation at 37^{o}C for 30 min. Following incubation, 3 μl of 0.1 mol L^{-1} EDTA was added followed by incubation for 10 min at 75^{o}C to inactivate the DNase. RNA concentration and quality in DNase treated samples were re-quantified using UV spectrophotometry at 260 nm and 280 nm.

Reverse transcription

Total RNA (1 μg) was used for first-strand cDNA synthesis. SuperScript III reverse-transcriptase (Invitrogen, Carlsbad, CA) was used according to the manufacturer's instructions. Reverse transcription reactions were primed with random hexamer oligonucleotides. Following reverse transcription, residual RNA was removed using RNase H (2 U). Each sample was spiked with approximately 10^5 copies of *in vitro* transcribed RNA (Alien QRT-PCR Inhibitor Alert; Stratagene, La Jolla, CA) as an external control. Reverse transcription was confirmed using control samples included with the reverse transcription kit. cDNA samples were stored at -20^{o}C for further analysis.

Primer design and verification

Fourteen genes corresponding to starch, protein, and lipid synthesis, kinases, transcription factors, and kernel maturation were selected for gene expression profiling (Table 1). If primer sequences were not available from literature sources, primers were designed using PrimerQuest software (Integrated DNA Technologies, Coralville, IA) and gene sequences from GenBank. Oligonucleotides were synthesized using standard desalting purification (Integrated DNA Technologies, Coralville, IA). Lyophilized primers were resuspended in DEPC treated water and adjusted to a concentration of 5 μmol L^{-1}. Prior to quantitative real-time PCR, primers were tested using standard PCR to verify that amplification products were of the correct size and that primer-dimers were not present. The PCR reaction began with 1 cycle of 3 min at 94^{o}C followed by 32 cycles at 94^{o}C for 30 s, 60^{o}C for 30 s, and 72^{o}C for 90 s. PCR products were visualized on a 2% agarose gel containing ethidium bromide.

Real-time quantitative PCR

SYBR Green chemistry was used for cDNA quantification. Reaction mixes consisted of 10 µl Brilliant II SYBR Green QPCR Mastermix (Stratagene, La Jolla, CA), 0.8 µl forward primer, 0.8 µl reverse primer, 0.3 µl ROX reference dye, 1 µl cDNA, and DEPC treated water to a total volume of 20 µl. The concentration of primers used was 5 µmol L^{-1}. A Stratagene Mx4000 Multiplex Quantitative PCR System was used for data acquisition (Iowa State University DNA Facility). All PCR reactions began with 1 cycle at 95°C for 10 min followed by 40 cycles at 95°C for 30 s and 1 min at 60°C. Following the final cycle of amplification, a dissociation program was run to check for non-specific product amplification and formation of primer-dimers. Products were incubated for 1 min at 95°C followed by ramping down to 55°C at a rate of 0.2°C s^{-1}. Forty-one cycles were completed in which the temperature was increased by 0.5°C $cycle^{-1}$ beginning at 55°C and ending at 95°C.

Five-fold serial dilutions ranging from 0.2 to 0.000032 relative concentration were run as a standard curve to estimate product amplification efficiency. C_t values were plotted versus the $\log_{10}$ values of the dilution values. The slope of the best-fit linear regression line was determined and used to calculate amplification efficiency (Equation 2). cDNA from a 12 DAP irrigated kernel sample was used as the calibrator sample. As such, relative transcript abundances were calculated based on this calibrator sample (Equation 3). Each sampling date was replicated at least twice per treatment. Each sample was analyzed for gene expression in duplicate.

$$\text{Efficiency} = 10^{\left(-1/\text{slope}\right)} - 1 \tag{2}$$

$$\text{Relative transcript abundance} = \frac{\left(1 + E_{GOI}\right)^{\Delta Ct_{GOI}}}{\left(1 + E_{\text{Normalizer}}\right)^{\Delta Ct_{\text{Normalizer}}}} \tag{3}$$

In Equation 3, E is the efficiency of amplification calculated by regression analysis of dilution curves for the gene of interest (GOI) and the normalizing gene. ΔC_t is the difference in C_t values between the calibrator sample and the unknown sample. The normalizing gene

is the *in vitro* transcribed RNA added to each sample prior to reverse transcription. The coefficient of variation for C_t values of this external reference between samples was approximately 2.3% indicating that reverse transcription efficiency was very consistent across samples.

Results and Discussion

Water stress imposed on developing kernels at 17 days post-pollination altered the pattern of kernel development. The impact of drought stress on kernel development was primarily a shortened duration of dry matter accumulation. Irrigated kernels gained dry matter at a rate of 5.2 mg per point of moisture content lost while water stressed kernels gained dry matter at a rate of 4.7 mg per point of moisture content lost ($P = 0.176$). Dry matter accumulation in the water stress treatment ended at about 48% MC while dry matter accumulation ended at approximately 40% in the irrigated treatment (Figures 1B; $P = 0.007$). These values for rate and duration of dry matter accumulation were calculated from a larger data set which included samples not used in this gene expression study (Chapter 2). When compared on a chronological basis (days post-pollination), kernel fresh weight, dry weight, and water content (mg per kernel) generally proceeded in a similar manner through 25 days post-pollination (Figures 2A-2C). By 25 days post-pollination, however, water content was 13.3% lower in the water stress treatment relative to irrigated kernels ($P = 0.023$). At 30 and 35 days post-pollination, all components of kernel weight were less in the water stress treatment. At physiological maturity, water stressed kernels had accumulated 186 mg dry weight while irrigated kernels had accumulated 227 mg dry weight. This represents a 17.9% reduction in dry weight accumulation relative to irrigated kernels ($P = 0.029$). These results are similar to those of our related study (Chapter 2) in which physiologically mature kernels from the water stress treatment were 21.2% smaller relative to irrigated kernels ($P < 0.0001$).

The impact of the terminal water deficit on kernel weight components was more evident when the pattern of development was normalized by kernel % MC (Figures 1A-1C). On sampling dates 25-35 days post-pollination, % MC values in the water stressed treatment were less than those in the irrigated treatment at equivalent dates. At 35 days post-pollination, the water content of irrigated kernels was 165 mg kernel^{-1} while the water

content of drought stressed kernels at 30 days post-pollination was 158 mg kernel^{-1} (Figure 2C; P = 0.290). The percent moisture content of water stressed kernels at 30 days post-pollination was approximately 47%. Similarly, the percent moisture content of irrigated kernels at 35 days post-pollination was approximately 42%. Therefore, when we used % MC as a developmental marker as suggested by Borrás and Westgate (2006), water stressed kernels at 30 days post-pollination and irrigated kernels at 35 days post-pollination were at nearly equivalent stages of development. These dates nearly coincide with the % MC values calculated for the termination of dry matter accumulation in each treatment. Evidently, the shortened duration of grain filling reflected the more rapid decline in kernel moisture content. It may also have reflected a depletion of assimilates to the kernel since water stressed kernels failed to accumulate dry matter to the same low moisture content observed in well watered kernels.

It was quite likely that the shorter duration of grain filling also signaled an early decline in gene expression. Several genes involved in storage product synthesis were selected for analysis since drought stress influences the total amounts of starch, protein, and oil accumulated as well as the relative concentrations of oil and protein within the kernel (Chapters 2 and 3). In related studies, we observed that the concentration of total kernel starch was not significantly affected by water stress during grain filling, while the numerical trend was for endosperm starch concentration to decrease with a concomitant increase in protein concentration. To examine the relationship between gene expression and kernel water relations, developmental patterns of gene expression were normalized on both % MC and dry matter accumulation scales. Figures 3A and 3B show the expression pattern of *Shrunken-2* (*Sh2*) versus declining kernel % MC and % of maximum dry matter accumulated. *Sh2* encodes for the large subunit of ADP-glucose pyrophosphorylase, the first committed enzyme in the starch biosynthetic pathway. Similar patterns of expression were observed using both scales of development. An approximately two-fold increase in relative mRNA abundance was measured at 15 days post-pollination in both treatments. This sampling point was prior to the initiation of the drought stress and coincided with the onset of rapid starch deposition. As such, an increase in transcriptional and metabolic activities related to primary storage reserve synthesis is expected (Doehlert and Lambert, 1991; Liu et

al., 2008). Expression of *Sh2* at 17 and 25 days post-pollination were similar to those measured at 12 days-post pollination. This is consistent with previous reports of post-translational regulation of ADP-glucose pyrophosphorylase by inorganic phosphate balance (reviewed in Hannah and James, 2008). The water deficit treatment did not appear to have a significant effect on *Sh2* expression at these sampling dates. At 30 and 35 days post-pollination, relative mRNA abundance for *Sh2* was less in the water stress treatment relative to the irrigated treatment. At physiological maturity, *Sh2* mRNA was present in both treatments indicating that expression of this gene was not a limiting factor to starch deposition within the endosperm of either water stressed or irrigated kernels. Although expression levels in both treatments appeared to decline rapidly in both treatments after maximum water content had been reached, it is not clear at what point, if any, *Sh2* mRNA level becomes limiting to starch accumulation.

Zein synthesis in developing maize endosperms is regulated primarily at the transcriptional level (Kodrzycki et al., 1989). As such, analysis of zein expression is useful for assessing differences in transcriptional profiles between treatments. The zein chosen for this study was the 19-kD α-zein B1 protein (*az19B1*), the most abundantly expressed of maize storage proteins (Woo et al., 2001). As with expression of *Sh2*, an increase in relative transcript abundance was measured at 15 days post-pollination. Water stress did not have a significant effect on 19-kD α-zein B1 expression from 12 to 25 days post-pollination. Another large peak in relative mRNA abundance was measured at approximately 30 days post-pollination in the irrigated treatment. Transcript abundance at this sampling date was nearly 17-fold higher relative to 12 days post-pollination consistent with previous reports of zein deposition late in kernel development. Transcript abundance in the water stress treatment was approximately 4-fold higher relative to 12 days post-pollination at this same sampling date. Woo et al. (2001), using *in situ* hybridization, reported that the *az19B1* transcript is most abundantly expressed in maize endosperm from 15 to 20 days post-pollination with lower, yet continued expression in the basal endosperm region at 25 days post-pollination. Although not entirely consistent with the results of Woo et al. (2001), these results suggest reduced accumulation of 19-kD α-zein in the endosperms of water stressed

kernels. This could partially explain the decreased kernel density and vitreousness measured in water stressed kernels by a related study (Chapter 2).

The *Opaque-2* (*O2*) gene encodes for a basic leucine zipper transcriptional activator that binds to the promoter region of the 22-kD α-zein (Schmidt et al., 1992). Mutants possessing the *o2* mutation possess a variety of unique phenotypes including perturbed zein accumulation (Landry and Delhaye, 2007), reduced kernel hardness (Lambert et al., 1969), and increased concentrations of essential amino acids such as lysine (Mertz et al., 1964). The expression pattern of *O2* shared many similarities with that of the 19-kD α-zein B1 transcript. An increase in relative mRNA abundance was observed in both treatments at 15 days post-pollination followed by a second peak of expression at 30 days post-pollination in the irrigated treatment. Together with the of 19-kD α-zein B1 expression data and the reduced density and vitreousness of water stressed kernels (Chapter 2), these results suggest that zein accumulation is impacted directly at the transcriptional level as a consequence of water stress during grain filling.

Conclusions

The yield and quality of maize grain are determined during kernel development. As such, understanding the mechanisms by which kernel development is perturbed in response to drought is important to providing rational strategies for crop improvement programs. It is not yet known if the expression of genes related to seed filling limit kernel size during stress conditions. The results of this study suggest that gene expression might not be limiting to seed filling even though dry matter ceases prematurely. Proper documentation of transcriptional mechanisms during kernel development requires a common basis for comparing profiles across genotypes and treatments. Kernel water content may provide such a basis, however, it is not yet clear if expression patterns of genes follow kernel water content. The results of this study indicate that in general, expression decreases gradually following maximum water content. Expression does not reach low levels, however, until after physiological maturity.

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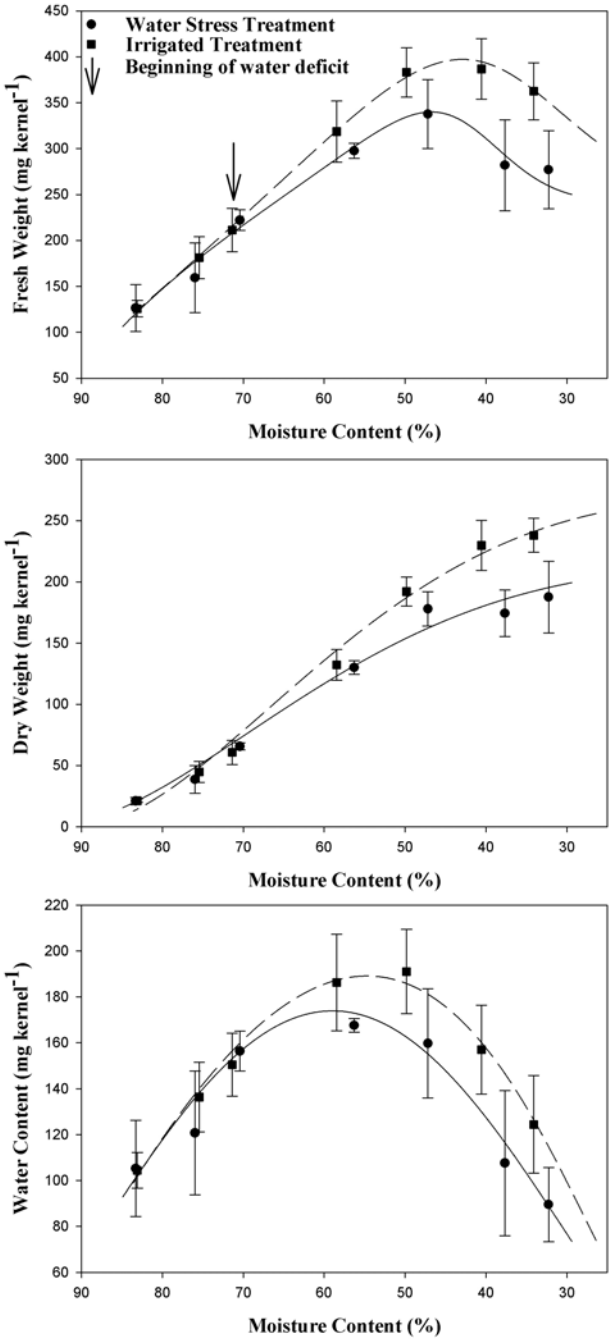
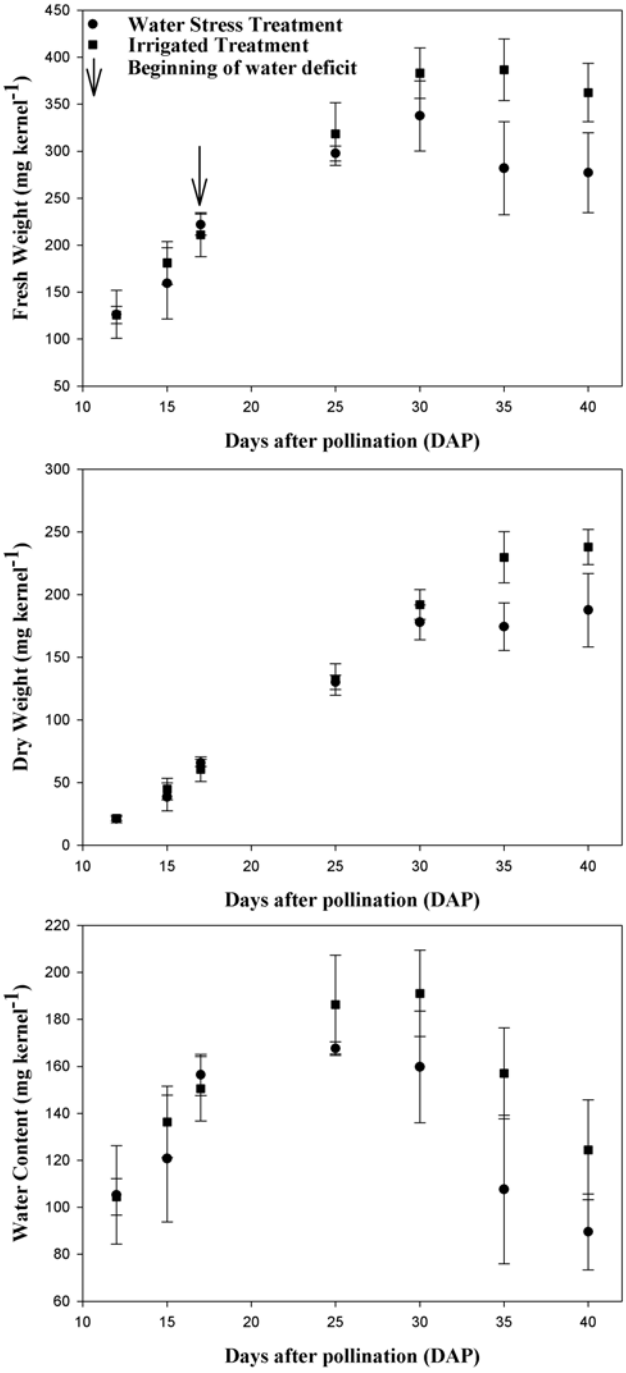
Table 1. Genes selected for expression profiling, primer sequences, approximate amplicon size, and references.

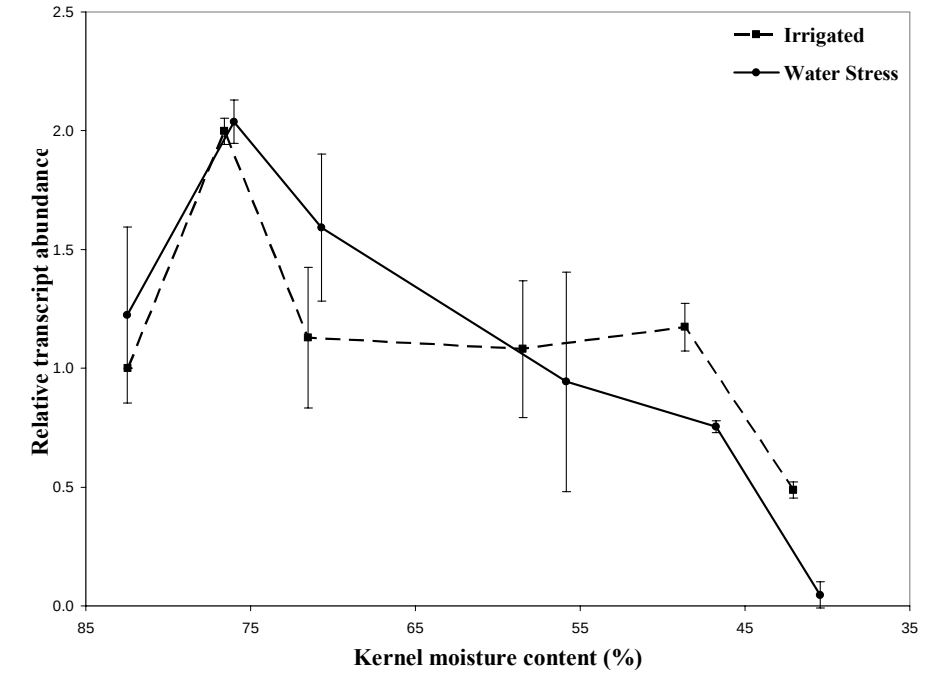
Gene:	Genbank Accession No.	Primer Sequences (5' to 3'):	Product size	Reference
cyPPDK1	NM_001112268	F: TAAACGATGCGGAGAAGCTCGTGA R: TAACATCATCCACCCAGGCCATGA	168	Mechin et al., 2007
ZmSPK1	AY722708	F: CATCGCTGCTGCATTCAAAGCCTA R: ACCGACCAGCATCACGTAAAGAGT	143	Zou et al., 2006
Sh2	AF544135	F: TATAGATCGGCTGCGTTTGCGTCT R: AGCGGCTCTTACCATACCAAGGTT	91	Whitt et al., 2002
Sh1	AF544103	F: TCAATGCCTCCTTTCCTCGTCCTT R: AAGCTCACCGTGCCCTTGTAGTTA	160	Whitt et al., 2002
Wx1	AF544073	F: TCGCGTCCTGCTGGTTCATTATCT R: TCATCCAGTTGATCTTCCGGCCTT	84	Whitt et al., 2002
O2	NM_001111951	F: ACAATCACACTGGAGGTAGCAGCA R: GCCTGCAGTTTGAGCGTGGTTATT	101	Gavazzi et al., 2007
Dbf1	NM_001112108	F: CAAGAGCAAGGCGATGCCAATCAA R: ACGGAACCTCGCTGAAATCCAACT	191	Kizis and Page, 2002
Dhn1	NM_001111949	F: ATGTGACAGGGACAGGGACAGTTT R: AGCCACTCGCAAGTGCTGTACTAT	128	-
15-kD beta-zein	AF371264	F: ATGATGATGGCGCAGAACATGC R: AATCAGTAGTAGGGCGGAATGGCA	113	Woo et al., 2001
27-kD gamma-zein	AF371261	F: TATGTGCTGTAGTATAGCCGCTGG R: ATTGCTCACACTGACACTGCCAC	112	Woo et al., 2001
19-kD alpha-zein B1	AF371269	F: TTGCCTCCTTATGCTCCTTGGTCT R: AAGGTAAGATGCCAGCTGCGATTG	188	Woo et al., 2001
ZmACO20	AY359575	F: CTCATCCTGCTGCTCCAGGACGAC R: TCCACGATACACGCATAACCACCGT	?	Gallie and Young, 2004
Acc1	NM_001111903	F: TCCCAACTCTTGCTTGGAGTGGAT R: TGCAACTGCTTCCTCTGTGGTAGT	119	Egli et al., 1995.
beta-tubulin		F: ACCAGATCGGCGCCAAGTTCT R: CATCATGTTCTTGGCATCCCACA	?	-

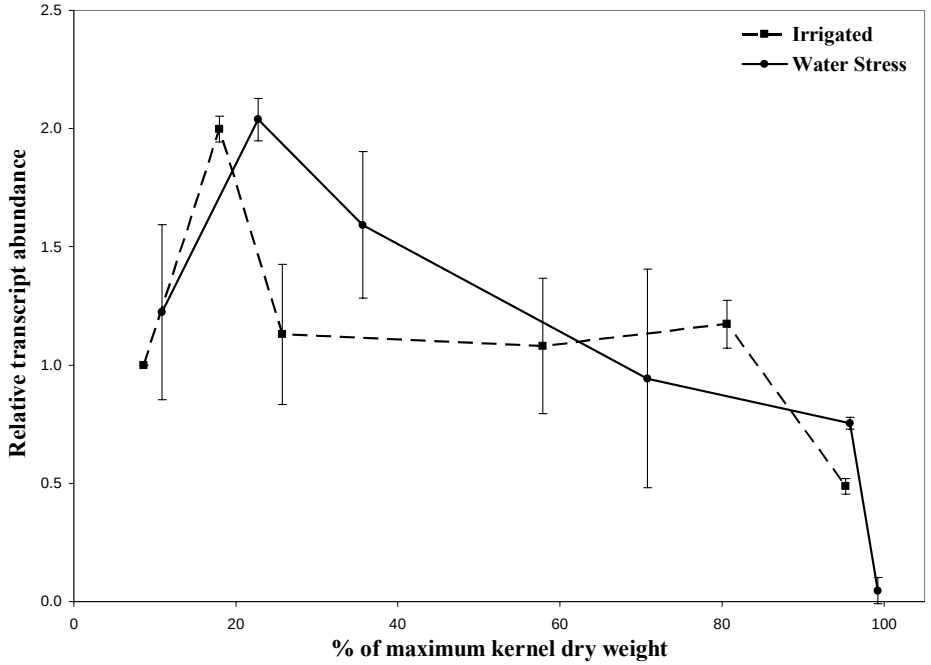


Figures 1A-1C. Progression of kernel fresh weight, dry weight, and water content relative to kernel moisture content. Data points are the means of samples taken at 12, 15, 17, 25, 30, 35, and 40 days after pollination. Each point is the mean ± SD of 2-15 replications.

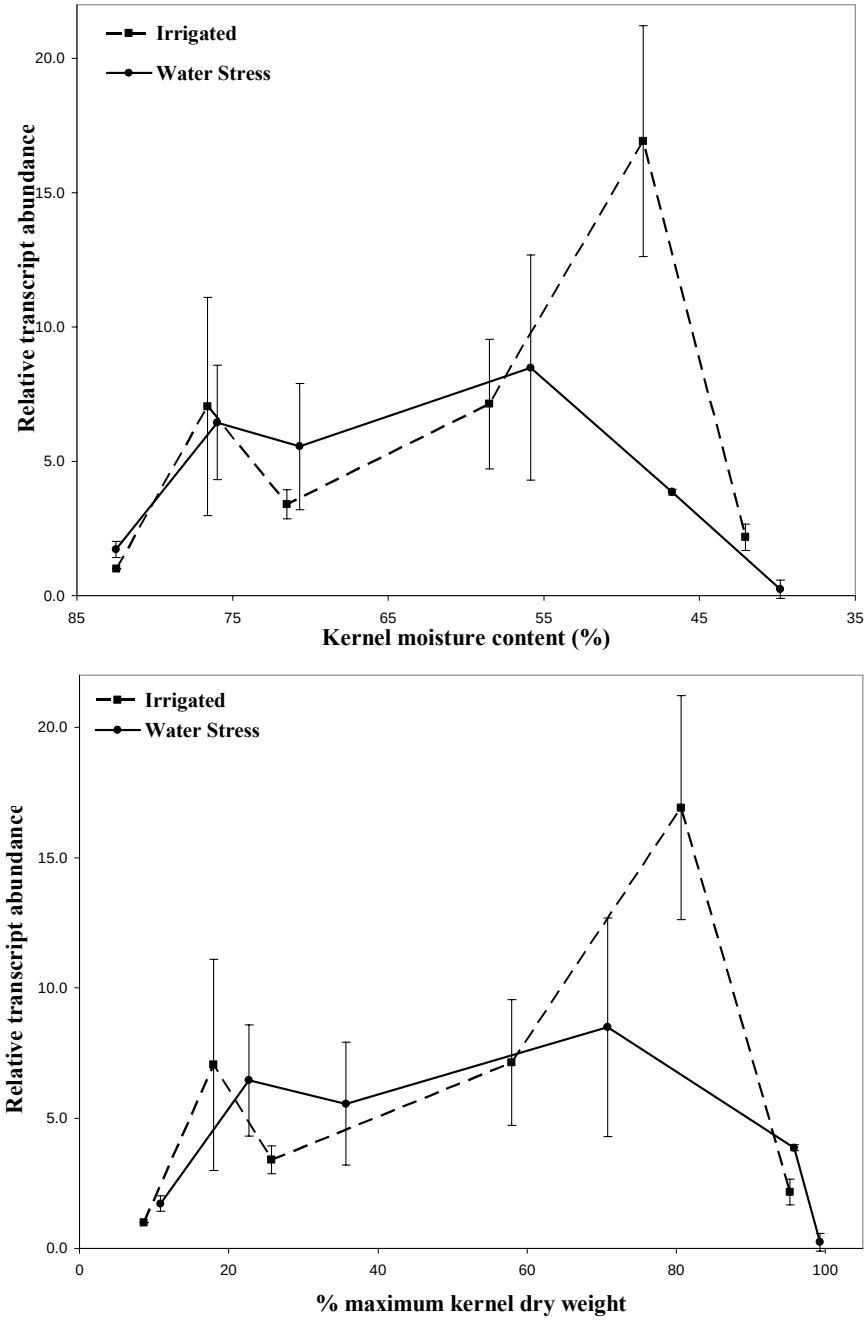


Figures 2A-2C. Progression of kernel fresh weight, dry weight, and water content relative to days after pollination. Each point is the mean ± SD of 2-15 replications.

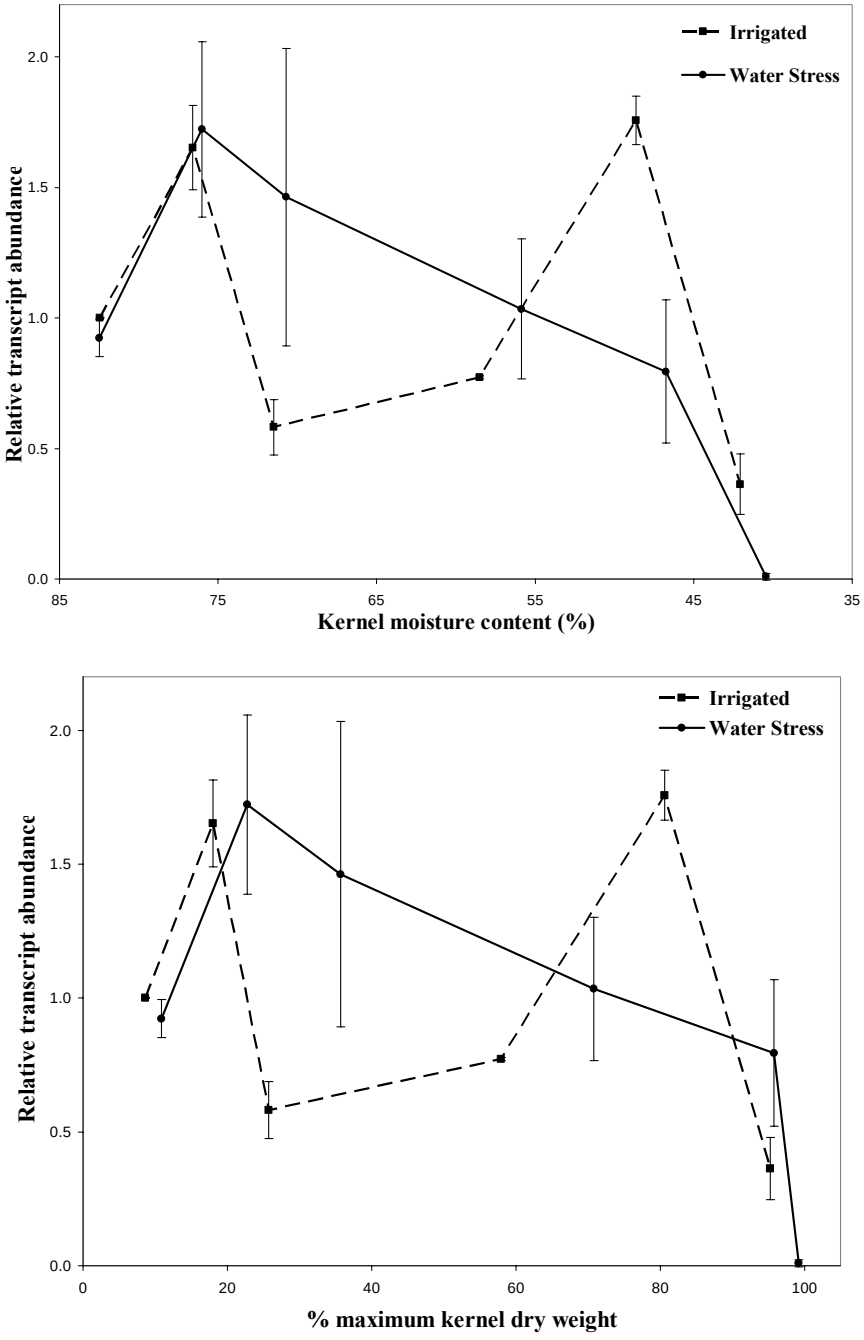




Figures 3A-3B. Gene expression profiles for *Sh2* (ADP-glucose pyrophosphorylase large subunit). Data points are the means for samples taken at 12, 15, 17, 25, 30, and 35 days after pollination. Error bars signify standard deviation of the mean. Relative transcript abundance based on 12 DAP irrigated endosperm cDNA. Transcript abundance versus kernel moisture content (3A) and versus % of maximum kernel dry weight (3B).



Figures 4A-4B. Gene expression profiles for *az19B1* (19-kD α-zein). Data points are the means for samples taken at 12, 15, 17, 25, 30, and 35 days after pollination. Error bars signify standard deviation of the mean. Relative transcript abundance based on 12 DAP irrigated endosperm cDNA. Transcript abundance versus kernel moisture content (4A) and versus % of maximum kernel dry weight (4B).



Figures 5A-5B. Gene expression profiles for *O2* (*Opaque2* transcription factor). Data points are the means for samples taken at 12, 15, 17, 25, 30, and 35 days after pollination. Error bars signify standard deviation of the mean. Relative transcript abundance based on 12 DAP irrigated endosperm cDNA. Transcript abundance versus kernel moisture content (5A) and versus % of maximum kernel dry weight (5B).

Chapter 5. General Conclusions

General Discussion

It is well understood that unfavorable environmental conditions during maize reproductive development are likely to impair grain yield through either reduced seed number or less total accumulation of dry matter within the kernel. The influence of a stress such as drought on grain quality, however, is not as well documented. With current interest in developing maize hybrids for specialized uses, it is important for breeders and physiologists to understand and develop strategies to improve and manage the quality of grain grown in diverse environments. The studies described in this thesis addressed how perturbations to kernel development as a result of late-season water stress affect the quality of grain for dry-grind ethanol production (Chapter 2), susceptibility to aflatoxin accumulation (Chapter 3), as well as how kernel development might be documented at the transcriptional level (Chapter 4). Although seemingly disparate in their foci, all are intrinsically related. For example, the dry-grind ethanol industry desires maize grain that is efficiently converted to ethanol, yet the characteristics that confer this trait might also increase the susceptibility of the grain to invasion by fungi such as *Aspergillus flavus*. This fungus produces a mycotoxin known as aflatoxin which is detrimental to human and animal health. When aflatoxin is present in grain used for ethanol, it is concentrated in the distiller's dried grains with solubles (DDGS) which are fed to livestock. Although ethanol conversion efficiency and yield might be increased, the safety of a valuable co-product could be diminished. Therefore, the two issues are intertwined and are ultimately consequences of genetic and environmental stimuli during kernel development. In a practical sense, developing maize germplasm that retains desirable characteristics across diverse environmental conditions will require a greater knowledge of the mechanisms responsible for specific phenotypes. Therefore, the ability to compare the gene expression of many genotypes and treatments using a reliable indicator of development such as kernel moisture content might shed new light into environmentally and developmentally specific gene transcription patterns. A tool such as this would be invaluable to plant breeders and crop physiologists.

Recommendations for Future Research

The research in Chapter 2 addressed grain composition in response to late-season chronic water stress. The conditions employed in this study might not necessarily be representative of field conditions in which the onset of drought could be more gradual. Additionally, plants in the field have a larger rooting environment to explore for water. Therefore, future research could investigate controlled water stress regimes under field conditions as well as the interaction of drought with other stresses such as high temperature. The second major focus of Chapter 2 was the susceptibility of starch to enzymatic hydrolysis. This characteristic is important to ethanol conversion efficiency and yield, yet these were not measured responses. Further work should complete the fermentation of starch from irrigated and water-stressed grain sources to determine if the measured differences in starch digestibility translate into significant differences in ethanol yield.

Chapter 3 focused on aflatoxin accumulation in mature kernels of a single genotype. Although the importance of α-amylase in fungal infection and aflatoxin synthesis is supported by a growing body of literature, this mechanism was not specifically addressed by Chapter 3. Future work should examine profiles of fungal growth, starch hydrolytic activity, and aflatoxin accumulation in developing kernels of multiple genotypes grown under optimal and drought conditions. Also, other proposed mechanisms for increased susceptibility to aflatoxin accumulation might be addressed.

Chapter 4 examined gene expression during kernel development of irrigated and water stressed B73 kernels. The major unresolved questions are i) do shifts in gene expression limit kernel development, and ii) does premature desiccation in water stressed plants trigger this? The expression patterns of three genes were reported, however, further expression profiles of genes fulfilling diverse roles in kernel development should also be documented.